

Spatial Variation in Host-Parasite Interactions in the Three-Spined Stickleback

Job de Roij, BSc.

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ABSTRACT

The role of parasites as agents of selection on their hosts has been well established, but less is known about how parasites facilitate divergence among host populations. In this thesis, I used the three-spined stickleback, *Gasterosteus aculeatus*, as a model organism to explore spatial variation in host-parasite interactions and the consequences for divergence of host traits. First, I established the extent of spatial variation in natural infection in the study system, North Uist, Outer Hebrides, Scotland, by conducting a survey of macroparasite communities in twelve freshwater lochs over two years. I found substantial geographic variation in parasite communities that was remarkably stable in time. Assuming that differences in parasite community composition correspond to differences in parasite-mediated selection, it suggests that North Uist stickleback populations experience divergent parasite-mediated selection that is consistent in time. Next, I carried out a series of artificial infection experiments with lab-reared sticklebacks from five populations using three widespread macroparasite species (*Gyrodactylus gasterostei*, *Diplostomum spathaceum* and *Schistocephalus solidus*), to assess geographic variation in parasite resistance and a component of the innate immune system, the respiratory burst response. There was significant variation among populations in resistance to *G. gasterostei* and *D. spathaceum*, and the innate immune response. To some extent the variation was related to natural infection levels, suggesting that divergent parasite-mediated selection may drive investment in these traits. Lastly, I conducted a growth experiment with the five stickleback populations and showed that there was significant population-level variation in juvenile growth rate, an important life history trait. In spite of considerable variation in all traits, I found no evidence for genetic trade-offs across populations between juvenile growth rate, and macroparasite resistance or the innate immune response. This thesis adds to a growing body of work that emphasises the importance of space in shaping host-parasite interactions.

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CONTRIBUTIONS

The work presented in this thesis is my own. However, a number of people made important contributions to individual chapters:

Chapter 3: Andrew MacColl designed the study. Andrew MacColl, Sonia Chapman and Sarah Forbes assisted with the collection and dissection of fish. Scott Young analysed the calcium concentration of water samples. Suzanne McGowan analysed the chlorophyll A concentration and DOC content of water samples.

Chapter 4: Phil Harris helped set up the large artificial infection experiment, aided with the identification of the *Gyrodactylus* species, and suggested the idea for the two-choice infection experiment.

For chapters 3-7, Andrew MacColl gave advice on statistical analysis and contributed comments to the discussion.

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CHAPTER 1: GENERAL INTRODUCTION

In this thesis I explore spatial variation in host-parasite interactions and examine the consequences for host population divergence. Below, I introduce the relevant concepts, using examples from vertebrate-macroparasite systems where possible. I begin by illustrating the role of parasites as agents of selection in host populations. I then consider what criteria must be met if parasite-mediated selection is to occur in a host population, the types of parasite-mediated selection and how parasite-mediated selection influences the evolution of parasite resistance. Finally, I emphasise the importance of space as a factor shaping host-parasite interactions.

1.1 Parasites as agents of selection

Parasitism is one of the most widespread lifestyles (Price, 1980). For every host species, there is probably at least one parasite species (Windsor, 1998; Poulin & Morand, 2000). Given that parasites form such a dominant component of ecological communities (Lafferty *et al.*, 2006), it is not surprising that they influence the ecology and evolution of their hosts. Parasites are known to regulate host population dynamics (Anderson & May, 1979; Hudson *et al.*, 1998; Tompkins *et al.*, 2002) impact host life history (Minchella, 1985; Perrin *et al.*, 1996; Agnew *et al.*, 2000) and manipulate host behaviour (Barnard & Behnke, 1990; Thomas *et al.*, 2005), to name just a few examples. For parasites to affect the evolution of their hosts, selection must be exerted on the genes underlying host phenotypes (Gillespie, 1975; Endler, 1986; Little, 2002). The potential of parasites as agents of evolutionary change and diversification in their hosts was first suggested by Haldane (1949). Since then, host-parasite interactions have been invoked to explain a range of evolutionary phenomena, including the evolution of sex (Jaenike, 1978; Hamilton, 1980; Howard & Lively, 1994), the maintenance of genetic polymorphism (Clarke, 1979) and sexual selection (Hamilton & Zuk, 1982; Penn & Potts, 1998), as well as ecological processes such as population regulation (Anderson & May, 1978; May & Anderson, 1978). Initially, many of these ideas were supported only theoretically, but there is now a growing body of empirical evidence in support of parasite-mediated selection driving host genetic change in the wild (Little, 2002; Woolhouse *et al.*, 2002; Summers *et al.*, 2003; Vamosi,

2005; Duffy & Forde, 2009). Parasites can affect the evolution of numerous host life history traits (Hochberg *et al.*, 1992; Møller, 1997; Fredensborg & Poulin, 2006), but the most interesting traits from a coevolutionary perspective are the defence traits that hosts have evolved in response to parasitism. Resistance to parasites may be mediated by a range of defence mechanisms (Rigby *et al.*, 2002), including behavioural avoidance (Hart, 1994; Karvonen *et al.*, 2004b), physical barriers (Wilson *et al.*, 2001) and perhaps most importantly, the immune system (Frank, 2002; Hedrick, 2002; Janeway *et al.*, 2004). Even if the mechanistic basis of resistance is unknown, it remains important to quantify genetic variation in parasite resistance in natural populations to understand how parasite-mediated selection may operate in the wild.

1.2 What are the requirements for parasite-mediated selection?

Two criteria must be met if parasite-mediated selection is to occur (Little, 2002). First, parasites must have detrimental effects on the growth, survival or reproduction (i.e. fitness) of their hosts. In vertebrates, this has been demonstrated extensively for a variety of species, ranging from viruses (Packer *et al.*, 1999; Telfer *et al.*, 2002) to endoparasitic helminths (Johnson *et al.*, 1999; Heins *et al.*, 2004) and ectoparasites (Lehmann, 1993; Richner *et al.*, 1993). Second, there must be genetic variation in resistance to parasites, such that infection is distributed unevenly among host individuals (Little, 2002). Asymmetric parasite distributions are common in the wild. Macroparasites, for instance, are typically aggregated within a host population; that is, a few individuals harbour disproportionately large parasite burdens relative to the rest of the population (Shaw *et al.*, 1998; Wilson *et al.*, 2002). However, variation in natural infection levels cannot be assumed to reflect genetic variation in resistance (Wilson *et al.*, 2002) because differences in exposure to parasites (Scott, 1991; Grosholz, 1994; Little & Ebert, 2000; Karvonen *et al.*, 2004a) and host condition (Beldomenico *et al.*, 2008) between host individuals can be equally important in determining parasite infection in the wild.

The most straightforward method for examining genetic variation in parasite resistance is to artificially infect a number of outbred individuals from a natural population under controlled conditions (Little, 2002). Rearing outbred individuals in a

uniform laboratory environment ('common garden') ensures that the contribution of environmental variation to the expression of traits is minimised. The advantage of this setup is that the observed variation among individuals/families can be assumed to have a genetic component (Lynch & Walsh, 1998). This approach has been successful for a number of invertebrate-microparasite (Ebert *et al.*, 1998; Tinsley *et al.*, 2006; Kraaijeveld & Godfray, 2008; Cory & Myers, 2009) and invertebrate-macroparasite (Henter & Via, 1995; Webster & Woolhouse, 1998; Hammerschmidt & Kurtz, 2005) systems. Thus far, most examples of genetic variation in resistance to parasites in vertebrates come from domesticated and laboratory populations (Wakelin, 1978; Kloosterman *et al.*, 1992; Sorci *et al.*, 1997; Gjedrem, 2000). Nevertheless, there are a several examples from natural populations. For example, Jackson & Tinsley (2005) found substantial family-level variation in resistance to a monogenean flatworm, *Protopolystoma sp.*, in a population of African claw-toed frogs, *Xenopus laevis*. Likewise, Uller *et al.* (2003) observed strong differences in resistance to a viral eye disease among full-sib families of Swedish common lizards, *Lacerta vivipara*, and Rauch *et al.* (2006a) found significant within-population variation in resistance to an eye fluke, *Diplostomum pseudospathaceum*, in three-spined sticklebacks. One of the aims of this thesis was to quantify variation in macroparasite resistance within vertebrate host populations, and in so doing, provide evidence for the potential of parasite-mediated selection.

Other studies have taken a more indirect approach to examining genetic variation in parasite resistance, by determining heritabilities of resistance of hosts in field experiments (Møller, 1990; Boulinier *et al.*, 1997; Brinkhof *et al.*, 1999; Smith *et al.*, 1999; Coltman *et al.*, 2001) or by using molecular tools to evaluate genotypes directly, particularly those hypothesised to play a role in disease resistance. A good example of the latter is genes of the major histocompatibility complex (Gilbert *et al.*, 1998; Paterson *et al.*, 1998; Langefors *et al.*, 2001; Wegner *et al.*, 2003).

1.3 Evidence for parasite-mediated selection in the wild

The presence of genetic variation in parasite resistance within a host population, as demonstrated by common garden experiments, can only provide evidence for the

potential of parasite-mediated selection. In fact, this variation is typically assumed in models of host-parasite coevolution (Anderson & May, 1982; Sorci *et al.*, 1997). It has been more difficult to show directly that parasite-mediated selection drives genetic change in natural host populations, i.e. changes in frequencies of alleles associated with resistance or those that are closely linked to resistance alleles (Little, 2002). The most convincing evidence in animals comes from invertebrate studies that track the temporal dynamics of host genotypes. For instance, Duncan & Little (2007) examined clone frequencies in a population of *Daphnia magna* before, during and after an epidemic of the bacteria *Pasteuria ramosa*, and found that genotypes that became more common after the epidemic were also more resistant to infection. Duffy & Sivers-Becker (2007) recorded a similar pattern for an epidemic of the fungus *Metschnikowia bicuspidata* in a population of *Daphnia dentifera*. These two examples suggest that parasites can drive directional selection for parasite resistance in natural host populations: episodes of selection were followed by an increase in the mean resistance of host genotypes. There is also some evidence for the occurrence of negative frequency-dependent coevolutionary dynamics in the wild. Negative frequency-dependent selection is a type of selection in which parasites track common host genotypes and decrease their fitness; this in turn confers an advantage on rare host genotypes, creating oscillations in gene frequencies. As this process is dependent on particular combinations of host and parasite genotypes, it can maintain genetic diversity for parasite resistance within a host population (Agrawal & Lively, 2002). Negative frequency dependent selection is at the heart of the Red Queen hypothesis, an idea that is frequently cited to explain the evolution of sex (Jaenike, 1978; Hamilton, 1980; Howard & Lively, 1994). Dybdahl & Lively (1998) conducted a five-year field study in a population of the snail *Potamopyrgus antipodarum* and found time lagged infection dynamics of the trematode *Microphallus* sp.; host clones that were recently common were overinfected relative to rare clones, suggesting that parasites can track and select against common host genotypes, in a frequency-dependent manner. In a recent study, Decaestecker *et al.* (2007) used dormant stages of *Daphnia magna* and *Pasteuria ramosa* from lake sediments to reconstruct coevolutionary dynamics. They also observed that parasites adapted rapidly to genetic changes in the host population (Decaestecker *et al.*, 2007). However, other studies have failed to find consistent changes in genotype frequencies or found changes that did match patterns of genetic variation in resistance from common garden

experiments (Henter & Via, 1995; Little & Ebert, 1999; Siemens & Roy, 2005). As a result, it has been difficult to show unequivocally that parasite-mediated selection is occurring in the wild.

1.4 The types of parasite-mediated selection and the evolution of parasite resistance

Parasites can exert different types of selection on their hosts. I have already mentioned directional selection and negative-frequency dependent selection. These two types of selection lead to fundamentally different coevolutionary dynamics and have different consequences for host diversification (Thompson, 1994; Agrawal & Lively, 2002; Woolhouse *et al.*, 2002; Summers *et al.*, 2003). Generally, directional selection is predicted to erode genetic variation in host traits whereas negative frequency dependent selection is thought to maintain it. However, the existence of other types of parasite-mediated selection, such as stabilising selection (Clayton *et al.*, 2005) and disruptive selection (Duffy *et al.*, 2008; Blanchet *et al.*, 2009) is becoming increasingly recognised. In stabilising selection, hosts with an intermediate level of the trait are selected for, whereas in disruptive selection, extreme values of the trait (high susceptibility and high resistance) are favoured. Stabilising selection and disruptive selection are predicted to decrease and increase host diversity, respectively (Endler, 1986; Duffy & Forde, 2009).

The type of parasite-mediated selection exerted, and hence the evolution of parasite resistance, is dependent on two key factors: costs of resistance and the genetic basis of the host-parasite interaction (May & Anderson, 1983; Frank, 1994b; Agrawal & Lively, 2002). It is often assumed that investment in parasite resistance is expensive and comes at the cost of investment in other life history traits. If trade-offs between parasite resistance and other fitness-related traits have a genetic basis, they can constrain the evolution of, and in turn maintain genetic variation in, parasite resistance (Sheldon & Verhulst, 1996; Coustau *et al.*, 2000; Rigby *et al.*, 2002). Theoretically, the presence or absence of trade-offs with parasite resistance, as well as the shape of trade-offs, can lead to different types of parasite-mediated selection, including directional selection for resistance (Bowers *et al.*, 1994; Frank, 1994a) and

stabilising selection (Boots & Haraguchi, 1999). There are several well-established examples of trade-offs between parasite resistance and other host life history traits in invertebrate-microparasite (Boots & Begon, 1993) and invertebrate-macroparasite (Kraaijeveld & Godfray, 1997; Webster & Woolhouse, 1999) systems, but examples in vertebrate-macroparasite interactions are currently lacking (although see e.g. Barber *et al.*, 2001). In Chapter 7 I test for the presence of trade-offs between macroparasite resistance and another life history trait, juvenile growth rate.

The genetic basis of host-parasite interactions also influences the evolution of parasite resistance (Sorci *et al.*, 1997; Woolhouse *et al.*, 2002). Two different genetic models have been used to characterise host-parasite interactions: the gene-for-gene (GFG) model and the matching allele (MA) model (Summers *et al.*, 2003; Lambrechts *et al.*, 2006b). In the simplest GFG model one parasite genotype is ‘universally virulent’ and can infect both the resistant and susceptible host genotypes; the resistant host genotype is resistant only to the avirulent parasite genotype (Flor, 1971; Thompson & Burdon, 1992). In contrast, in the simplest MA model, infection occurs only when the virulence alleles of the parasite match the resistance alleles of the host (Frank, 1994b; Agrawal & Lively, 2002). Host-parasite interactions following the MA model are associated with strong host genotype by parasite genotype interactions and a high degree of specificity (Summers *et al.*, 2003). As a result, frequency-dependence is inherent in MA models and negative frequency-dependent parasite-mediated selection is likely to occur. Host-parasite interactions following the GFG model, on the other hand, are associated with low specificity and low frequency-dependence (Summers *et al.*, 2003). GFG models typically involve a few major genes of the host and parasite (Thompson & Burdon, 1992), whereas MA models may involve more host and parasite genes of smaller effect (Agrawal & Lively, 2002). Rather than forming a dichotomy, GFG and MA models comprise two ends of a continuum of host-parasite coevolutionary dynamics (Parker, 1994; Agrawal & Lively, 2002). The two aspects, the genetic basis of host-parasite interactions and costs of resistance, are linked: GFG models often assume a high cost of resistance (to stop the resistant host genotype from going to fixation), while MA models often assume either a low cost of resistance or no cost at all (Frank, 1994b). Furthermore, specific and non-specific forms of host defence may differ in costliness (Frank, 2000; Jokela *et al.*, 2000; Moret, 2003; Schmid-Hempel & Ebert, 2003).

1.5 Spatial variation in parasite-mediated selection and host divergence

Until now, I have considered parasite-mediated selection solely in the context of generating and maintaining variation within the same host population. However, this neglects an important feature of host-parasite interactions: they are spatially variable (Frank, 1991; Thompson, 2005). Geographically distinct host populations are likely to experience divergent host-parasite coevolution as a result of spatial variation in parasite distributions (Jokela & Lively, 1995; Behnke *et al.*, 2004; Poulin, 2007b), differences in genetic constitution of hosts and parasites (Lively, 1989; Grosholz, 1994; Henter & Via, 1995; Ebert *et al.*, 1998; Thrall *et al.*, 2002; Prugnolle *et al.*, 2006), variation in the abiotic environment (Bedhomme *et al.*, 2004; Mitchell *et al.*, 2005), and their interaction (Tetard-Jones *et al.*, 2007; Laine, 2009). Although most work on host-parasite interactions has focused on the temporal dynamics of parasite-mediated selection (Dybdahl & Lively, 1998; Decaestecker *et al.*, 2007; Gaba & Ebert, 2009), parasite-mediated selection is potentially even more variable in space than it is in time (Duffy & Forde, 2009; Wolinska & King, 2009). Moreover, studying multiple host populations can inform our understanding of temporal aspects of host-parasite interactions, if different host populations are at different stages of a coevolutionary cycle (Frank, 1991; Morand *et al.*, 1996; Lively, 1999). In such a scenario coevolutionary dynamics in space and time may be qualitatively similar (Woolhouse *et al.*, 2002; Gandon *et al.*, 2008; Wolinska & Spaak, 2009).

Both the strength and type of selection exerted by parasites on their hosts are likely to vary spatially. As explained above, directional selection is predicted to deplete genetic variation for traits within populations, whereas negative frequency-dependent selection is thought to have the opposite effect. However, this may not be the case when comparing different host populations (Rieseberg *et al.*, 2002; Summers *et al.*, 2003). For example, Buckling & Rainey (2002b) examined the evolution (diversity) of bacterial populations of *Pseudomonas fluorescens* in the presence and absence of a bacteriophage and found that selection imposed by the phage significantly increased among-population diversity, whereas within-population diversity was reduced. Given that parasite-mediated selection in bacterial populations is predominantly directional (via selective sweeps) (Chao *et al.*, 1977; Buckling & Rainey, 2002a), this supports a role

for directional selection as a diversifying force *among* populations. Moreover, if the costs associated with maintaining parasite resistance (Sheldon & Verhulst, 1996; Rigby *et al.*, 2002) vary among populations, either due to ecological differences, such as resource availability, or differences in the genetic relationships between life history traits, directional parasite-mediated selection can further contribute to divergence among host populations (Summers *et al.*, 2003; Duffy & Forde, 2009).

One of the most common spatial patterns in host-parasite interactions is local adaptation (Kaltz & Shykoff, 1998; Dybdahl & Storfer, 2003; Greischar & Koskella, 2007; Hoeksema & Forde, 2008). Host local adaptation is defined as the increased resistance of hosts to sympatric parasites compared to allopatric parasites, whereas parasite local adaptation is defined as the increased infectivity of parasites in sympatric hosts compared to allopatric hosts (Kawecki & Ebert, 2004). Local adaptation is tested experimentally by carrying out cross-infection or reciprocal transplant experiments with a number of host and parasite populations. Theory predicts that parasites should be more frequently locally adapted to their host populations than *vice versa*, due to their shorter generation times, higher fecundity and higher rates of migration (Lively, 1999; Gandon, 2002; Nuismer, 2006). Empirical work generally bears out this prediction (Parker, 1985; Lively, 1989; Ebert, 1994). However, there are several examples in the literature of host local adaptation (parasite local *maladaptation*). For example, Kaltz *et al.* (1999) found that the plant *Silene latifolia* was significantly more resistant to a fungal pathogen, *Microbotryum violaceum*, from sympatric rather than allopatric populations. Similarly, Gasnier *et al.* (2000) documented that *Lymnaea stagnalis* had a lower abundance of metacercariae of *Fasciola hepatica* when exposed to a sympatric strain than when exposed to an allopatric strain. The majority of local adaptation studies are restricted to plants and invertebrates (Kaltz & Shykoff, 1998; Lajeunesse & Forbes, 2002; Greischar & Koskella, 2007). Nonetheless, a few studies have been conducted in natural vertebrate-parasite systems and they support both parasite local adaptation (Ballabeni & Ward, 1993; Jackson & Tinsley, 2005) and host local adaptation (Oppliger *et al.*, 1999; Kalbe & Kurtz, 2006) scenarios.

Local adaptation may be seen as indirect evidence for the potential of host-parasite coevolution as it demonstrates the presence of genetic variation in both host traits

(resistance) and parasite traits (infectivity) in natural populations. It partly explains how variation in these traits can be maintained among populations (Summers *et al.*, 2003; Laine & Tellier, 2008). However, its absence does not indicate that host-parasite coevolution is not occurring. Instead, spatial patterns of host-parasite interactions may be more complex. In fact, the detection of local adaptation may depend strongly on the spatial scale at which patterns are investigated (Kaltz & Shykoff, 1998; Laine, 2005; Gandon *et al.*, 2008; Cogni & Futuyma, 2009). The 'geographic mosaic of coevolution' (Thompson, 1999; 2005) provides a framework for examining spatial heterogeneity in host-parasite interactions. It postulates that three key processes govern coevolutionary dynamics: coevolutionary hot and cold spots, selection mosaics and trait remixing (Gomulkiewicz *et al.*, 2007). These processes are ultimately responsible for generating the three patterns that define a geographic mosaic: spatial variation in host and parasite traits, mismatching of these traits (local adaptation and maladaptation), and few traits that have coevolved at the species level (Thompson, 1999; 2005). The theory has the potential to explain the evolution of complex spatial patterns of host resistance to parasites because it incorporates both biotic (parasite) and abiotic (environment) factors. Yet, verifying the three central processes empirically, and hence providing evidence for the existence of geographic mosaics, has proven difficult (Gomulkiewicz *et al.*, 2007). For example, spatial variation in parasite resistance may simply be the product of differences among populations in initial genotype frequencies (Morand *et al.*, 1996) rather than coevolutionary hot spots and cold spots or selection mosaics, thereby giving the impression of a geographic mosaic.

A useful method for detecting coevolutionary host spots and cold spots is to conduct comparative spatial analyses of parasite resistance phenotypes of hosts (Berenbaum & Zangerl, 1998; Laine, 2006; Gomulkiewicz *et al.*, 2007). In particular, assaying resistance to parasites among a number of geographically distinct host populations may offer insights into divergent host-parasite interactions. Local adaptation studies, by definition, examine geographic variation in parasite resistance. However, a limitation of such studies is that they require a factorial experimental design, that is, reciprocal cross-infection experiments must be carried out for all host-parasite combinations. This setup quickly becomes very labour-intensive and is restrictive in terms of providing a general overview of spatial variation in parasite resistance if few host-parasite combinations are used. Instead, lab-reared individuals from several

natural host populations can be exposed to a standard parasite genotype (or population) in common garden conditions to determine (genetically-based) geographic variation in resistance. This approach has been used successfully for a range of different invertebrate-parasite interactions (Kraaijeveld & van Alphen, 1995; Mucklow *et al.*, 2004; Tinsley *et al.*, 2006; Corby-Harris & Promislow, 2008; Cory & Myers, 2009). Again, there are fewer examples of common garden studies examining between-population variation in resistance to microparasites or macroparasites in vertebrates (but see e.g. Uller *et al.*, 2003; Jackson & Tinsley, 2005; Kalbe & Kurtz, 2006; Tobler & Schmidt, 2010). Hence, another aim of this thesis was to assess geographic variation in macroparasite resistance among multiple host populations. To address this question, I carried out a series of infection experiments with three macroparasite species (see Section 2.3). Others have used geographic variation in the diversity of immune genes, such as MHC genes, to infer divergent parasite-mediated selection (Miller *et al.*, 2001; Ekblom *et al.*, 2007; Alcaide *et al.*, 2008; Matthews *et al.*, 2010a). However, constitutive expression of immune components is likely to be equally variable in space, and this variation may also have a genetic basis (Sanjayan *et al.*, 1996; Lindström *et al.*, 2004; Cornet *et al.*, 2009). In Chapters 5 and 6, I evaluate variation in the innate immune response among host populations.

Although we are beginning to appreciate the role of parasites as agents of selection and diversification in single host populations (Summers *et al.*, 2003; Duffy & Forde, 2009), it is less clear how parasites drive divergence among populations (Buckling & Rainey, 2002b; Vamosi, 2005). As mentioned before, common garden experiments incorporating multiple host populations can identify patterns of spatial variation in parasite resistance and immune response and reveal divergence in these traits. However, an understanding of geographic variation in host ecology is likely to help explain and interpret these patterns (Sadd & Schmid-Hempel, 2009). For example, by characterising differences in parasite community composition among populations and integrating these data with measures of spatial variation in parasite resistance and immune response, it may be possible to determine whether the variation in parasite resistance and immune response is the result of adaptation to local parasite communities. In other words, is investment in these traits optimised according to the local rate of parasite exposure? The link between natural infection and experimental resistance or immune response across populations has received surprisingly little

empirical attention (but see e.g. Lindström *et al.*, 2004; Scharsack *et al.*, 2007a; Corby-Harris & Promislow, 2008; Hasu *et al.*, 2009). In Chapters 4-6, I aim to link spatial variation in macroparasite resistance and immune response to spatial variation in natural parasite infection.

1.6 Thesis outline and main aims of each chapter

Chapter 2 – Host study species, study system, and parasite species

I give a brief description of the host study species (the three-spined stickleback, *Gasterosteus aculeatus*), the study system (North Uist, Outer Hebrides, Scotland), and the three macroparasite species used in the artificial infection experiments (*Gyrodactylus gasterostei*, *Diplostomum spathaceum* and *Schistocephalus solidus*).

Chapter 3 – The relative contribution of spatial and temporal variation to macroparasite community composition in sticklebacks

I conducted a survey of macroparasite communities in 12 freshwater stickleback populations in two consecutive years to examine whether: 1) there is variation in parasite communities among populations, 2) this variation is consistent across years, and 3) spatial differences in parasite community composition can be explained by differences in geomorphological or physicochemical habitat characteristics.

Chapter 4 – Divergent resistance to a monogenean flatworm among stickleback populations

I carried out artificial infection experiments with the fin ectoparasite *Gyrodactylus gasterostei* in five lab-reared stickleback populations to investigate: 1) variation in resistance to *G. gasterostei* among populations, and 2) the relationship between natural *Gyrodactylus* infection and resistance to *G. gasterostei*.

Chapter 5 – The relationship between parasite resistance and the innate immune response across stickleback populations

I conducted an artificial infection experiment with the eye fluke *Diplostomum spathaceum* in five lab-reared stickleback populations to examine: 1) variation in resistance to *D. spathaceum* among populations, 2) variation in the innate immune response to *D. spathaceum* among populations, 3) the relationship between the innate

immune response and *D. spathaceum* resistance across populations, 4) the relationship between natural infection *Diplostomum* infection, and resistance to *D. spathaceum* or the innate immune response and 5) the effect of *D. spathaceum* infection on stickleback growth.

Chapter 6 – Examining variation in parasite resistance and the innate immune response to a tapeworm among stickleback populations

I carried out an artificial infection experiment with the body cavity tapeworm *Schistocephalus solidus* in five lab-reared stickleback populations to investigate: 1) variation in resistance to *S. solidus* among populations, 2) variation in the innate immune response to *S. solidus* among populations, 3) whether variation in resistance is linked to natural *S. solidus* infection and 4) the effect of *S. solidus* infection on stickleback growth and energy status.

Chapter 7: Are there trade-offs between juvenile growth rate and parasite resistance or innate immune response in sticklebacks?

I conducted a growth experiment with five lab-reared stickleback populations to examine variation among populations in juvenile growth rate, an important life history trait. Then, integrating data from Chapters 4 and 5, I looked for evidence of trade-offs, at the level of full-sib families across populations, between juvenile growth rate, resistance to *G. gasterostei* and *D. spathaceum*, and the innate immune response. This was possible because the same five stickleback populations were used in the growth experiment and the infection experiments.

Chapter 8 – General discussion

I integrate the findings from all data chapters, discuss their implications and limitations, and suggest areas for further work.

CHAPTER 2: HOST STUDY SPECIES, STUDY SYSTEM & PARASITE SPECIES

2.1 Host study species: the three-spined stickleback, *Gasterosteus aculeatus*

The three-spined stickleback, *Gasterosteus aculeatus* (Fig. 2.1), is a small teleost fish distributed throughout much of the northern hemisphere (Wootton, 1976; Bell & Foster, 1994). It has a long history as a model organism (Wootton, 2009) and has been used widely to investigate behaviour (Tinbergen, 1951; Huntingford, 1976; Bakker, 1986) and to address ecological and evolutionary questions (Heuts, 1947; Hagen, 1967; Schluter & McPhail, 1992; Schluter, 2000; McKinnon *et al.*, 2004; Bolnick, 2004). More recently, with the publication of the stickleback genome, sticklebacks have also shed light on the molecular genetics of adaptation and development (Peichel *et al.*, 2001; Cresko *et al.*, 2007; Colosimo *et al.*, 2005). The main reason this species has received so much attention from evolutionary biologists apart from being common and widely distributed is that it has recently undergone an adaptive radiation. Following the glacial retreat at the end of the last ice age approximately 10,000 years ago, ancestral marine sticklebacks have repeatedly colonised freshwater habitats (Bell & Foster, 1994; McKinnon & Rundle, 2002). Adaptation to these novel environments led to the divergence of numerous traits, including morphology (Shapiro *et al.*, 2004), life history (Baker, 1994; Schluter, 1995) and behaviour (Boughman, 2001; Dingemanse *et al.*, 2007). The large number of populations that have evolved in parallel as a result of this radiation provides an ideal system for examining the role of ecology in diversification and speciation (McKinnon *et al.*, 2004), especially considering that parallel evolution forms some of the most convincing evidence for the action of natural selection in the wild (Rundle *et al.*, 2000; Schluter, 2000). An additional benefit of the three-spined stickleback is that viable hybrids can be produced readily between sympatric populations that no longer interbreed, such as the limnetic/benthic stickleback species pairs (Bell & Foster, 1994), or between allopatric populations that have diverged substantially. Therefore, comparisons of hybrid and parental populations can offer insights into the ecological mechanisms contributing

to reproductive isolation (Hatfield & Schluter, 1999; Vamosi & Schluter, 2002; Vines & Schluter, 2006) as well as the genetic basis of complex traits (Colosimo *et al.*, 2005; Albert *et al.*, 2008).

To date, most ecological and evolutionary studies of sticklebacks have focused on how resource competition and polymorphism (Gray & Robinson, 2002; Svanback & Bolnick, 2007; Matthews *et al.*, 2010b), and predation (Vamosi, 2002; Rundle *et al.*, 2003; Reimchen & Nosil, 2004; Marchinko, 2009) drive population divergence. However, the last decade has seen an increasing interest in host-parasite interactions of sticklebacks and their consequences for host divergence. The stickleback fauna is well characterised (Chappell, 1969; Wootton, 1976; Barber, 2007; Zander, 2007) and spatial variation in parasite communities has been recorded (Kalbe *et al.*, 2002; MacColl, 2009a). Moreover, for certain common stickleback parasites, such as the body cavity cestode *Schistocephalus solidus* and the eye fluke *Diplostomum spathaceum*, the negative impacts on host fitness are known (Brassard *et al.*, 1982; Owen *et al.*, 1993; Barber *et al.*, 2008; Heins & Baker, 2008; Barber & Scharsack, 2010). As a result, sticklebacks exposed to these virulent parasites may be under strong selection to evolve defence mechanisms. The German lake-river stickleback system has been the most thoroughly studied in terms of host-parasite interactions, with a strong emphasis on the MHC genes (Wegner *et al.*, 2003; Kurtz *et al.*, 2006; Rauch *et al.*, 2006b; Eizaguirre *et al.*, 2009b; Kalbe *et al.*, 2009). In this system, variation in parasite communities (Kalbe *et al.*, 2002) appears to shape differential parasite resistance (Kalbe & Kurtz, 2006), immune response (Scharsack *et al.*, 2007a) and MHC allelic diversity (Wegner *et al.*, 2003). The well-established limnetic-benthic species pairs in British Columbia have also attracted interest with regards to host-parasite coevolution (MacColl, 2009a; 2009b; MacColl & Chapman, 2010; Matthews *et al.*, 2010a). However, we still have a limited understanding of how parasites can drive stickleback divergence.



Figure 2.1. The study organism, the three-spined stickleback, *Gasterosteus aculeatus*. This fish is a lab-reared fish from Loch Hosta, North Uist. Photo by Andrew MacColl.

2.2 Study system: North Uist, Outer Hebrides, Scotland

North Uist is a small island (~300km²) in the Outer Hebrides, Scotland. The landscape comprises a vast network of freshwater and saltwater lochs (Fig. 2.2), the majority of which harbours three-spined sticklebacks. Since most of the lochs are not interconnected, these stickleback populations may be considered to be geographically isolated and evolutionarily independent. The phylogeographic history of North Uist sticklebacks remains to be studied, but preliminary microsatellite data (S. Coyle, personal communication) indicate that some populations have been separated long enough to have diverged substantially. The extensive morphological variation, in terms of body size, body shape and armour traits, among freshwater stickleback populations on North Uist (Giles, 1983b) certainly suggests that there has been divergence. However, with the exception of a few studies published in 1980's (Giles, 1983b; Giles & Huntingford, 1984), very little is actually known about the ecology and evolution of sticklebacks in this system, let alone the impact of parasites on stickleback evolution.

Freshwater lochs on North Uist are characterised by substantial environmental variation, most notably a cline in alkalinity/acidity that runs from the west side to the east side of the island (Giles, 1983b), but conductivity and water temperature also vary spatially (A. MacColl, unpublished data). Alongside physicochemical differences, freshwater lochs differ considerably in size and depth, and there is spatial variation in phytoplankton and zooplankton densities (A. MacColl, unpublished data). Given these environmental differences and the large number of replicate stickleback populations, North Uist forms an excellent system in which to study the ecology and evolution of

spatial variation in host-parasite interactions. The stickleback populations examined in this thesis were chosen to cover a wide geographical and environmental range, with the aim of gaining a broad overview of spatial variation in host-parasite interactions. All but one of the populations are freshwater populations; one saltwater population was also included in the infection and growth experiments (Chapters 4-7).

2.3 Parasite species: *Gyrodactylus gasterostei*, *Diplostomum spathaceum* and *Schistocephalus solidus*

In Chapter 4-6, artificial experiments were carried out with three common stickleback macroparasite species: *Gyrodactylus gasterostei*, *Diplostomum spathaceum* and *Schistocephalus solidus*. Below, I give a brief description of the life cycle and life history of each parasite species.

2.3.1 *Gyrodactylus gasterostei*

Gyrodactylus gasterostei is a monogenean ectoparasite that infects the fins and body of freshwater three-spined sticklebacks (Glaser, 1974; Harris, 1985). Like most gyrodactylids, this species is viviparous and lacks a specific transmission stage. Worms reproduce directly on the host and have a short generation time (2-4 days depending on the water temperature). This short generation time is achieved in part due to their unusual mode of reproduction. Gyrodactylids give birth to a fully grown daughter which itself contains a developing embryo *in utero*, akin to “Russian Dolls” (Bakke *et al.*, 2007). As a result, growth of the parasite population can be extremely rapid (Fig. 2.3). Although gyrodactylids can reproduce both sexually and asexually, reproduction in *G. gasterostei* tends to be predominantly asexual (Harris, 1998). *Gyrodactylus* transmission between hosts occurs when fish come into close contact.

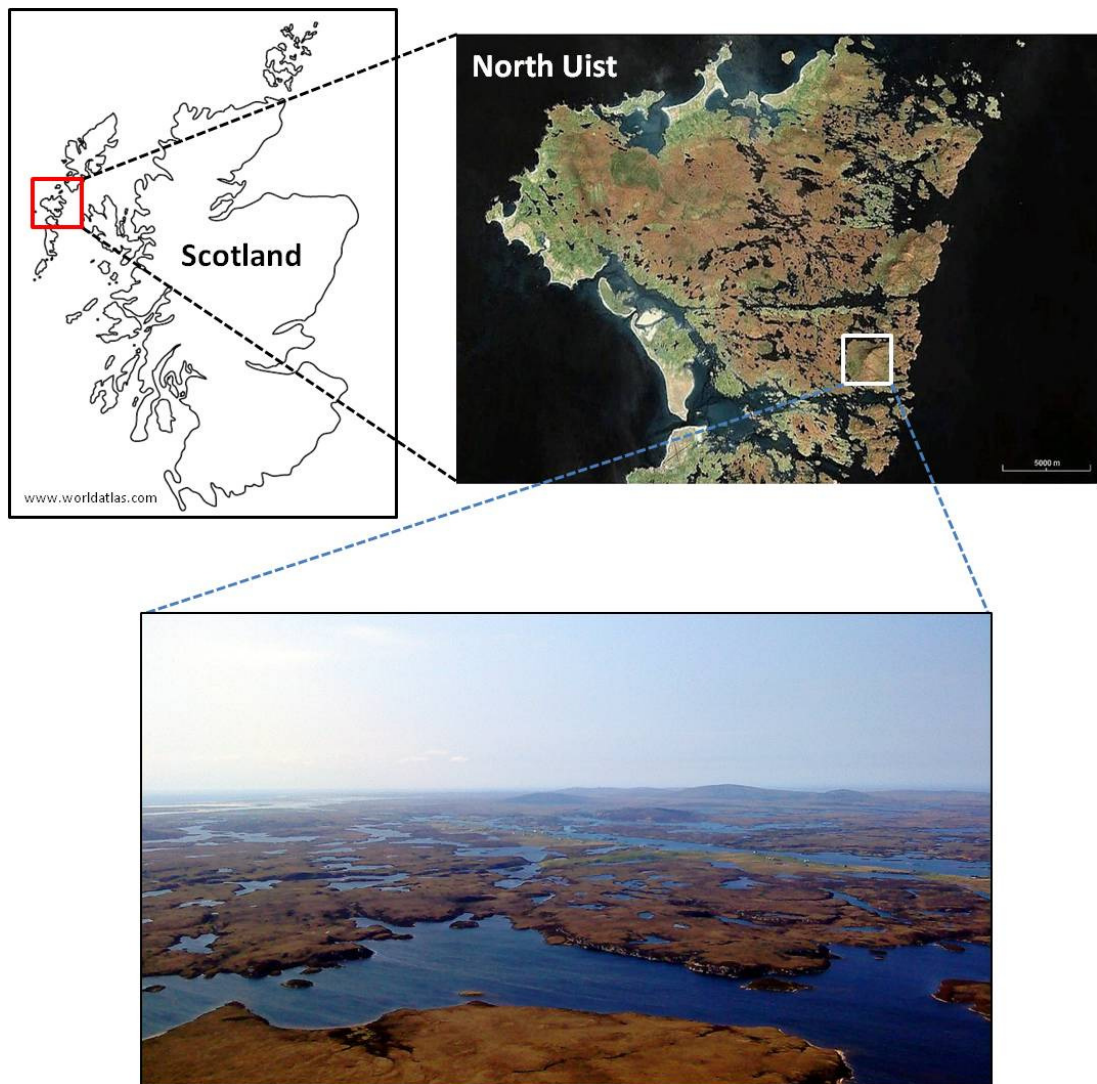


Figure 2.2 The study system, North Uist, Outer Hebrides, Scotland. The photo shows the view from Eaval to the North West, May 2008, and depicts the interlocking matrix of water bodies. The loch (Obisary) in the foreground is flooded by the sea at spring tides and is brackish. Lochs in the middle distance are acidic freshwater. The Atlantic can just be seen in the distance in the upper left. The satellite image of North Uist was obtained from WorldWind (NASA; <http://worldwind.arc.nasa.gov/>).



Fig. 2.3 A scanning electron micrograph of a heavy *Gyrodactylus* infection. Photo by K. Kvalsvik, reproduced from Bakke et al. (2007)

2.3.2 *Diplostomum spathaceum*

Diplostomum spathaceum is a widely distributed digenean trematode that uses a range of fish species, including the three-spined stickleback, as a second intermediate host in its three-host life cycle (Chappell, 1995; Fig. 2.4). Adult worms mature in the gut of a fish-eating bird, the definitive host, and reproduce sexually to produce eggs. Eggs are deposited in the water along with bird faeces. Here, they hatch to release miracidia, the first free-swimming larval stage that locates, penetrates and infects snails (usually *Lymnaea stagnalis*), the first intermediate hosts. In the snail, miracidia develop into sporocysts that undergo asexual reproduction to produce cercariae, the second free-swimming larval stage. Fish become infected through exposure to cercariae shed from infected snails. Cercariae penetrate the fish gills and skin and migrate to the eye lens, where they develop into metacercariae. Metacercariae remain in the lens until the infected fish is eaten by a bird (e.g. a gull). In the bird gut, metacercariae develop into adult worms, thus completing the life cycle (Chappell, 1995).

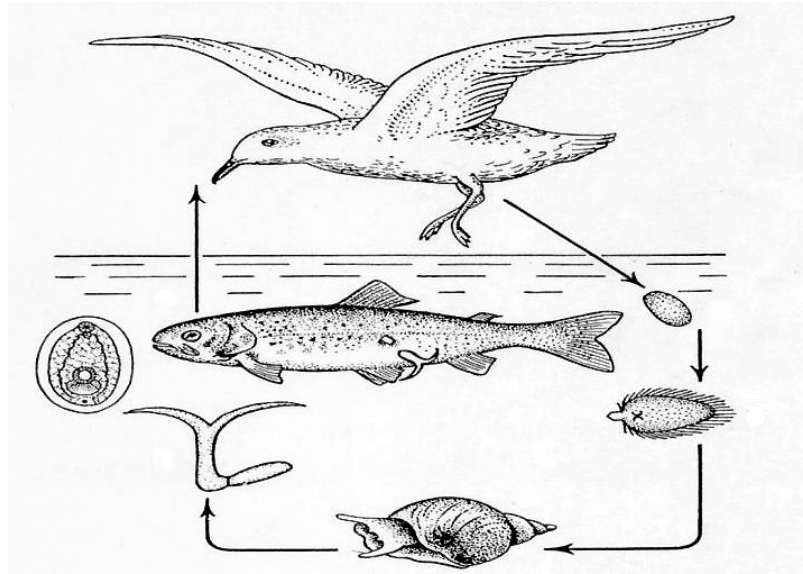


Fig. 2.4. The life cycle of *Diplostomum spathaceum*. Figure from Dogiel et al. (1961).

2.3.3 *Schistocephalus solidus*

Schistocephalus solidus is a pseudophyllidean cestode that regularly infects three-spined sticklebacks, the second and only obligate intermediate host in its three-host life cycle (Barber & Scharsack, 2010; Fig. 2.4). In the gut of a fish-eating bird, the definitive host, adult worms mature and reproduce either sexually or asexually to produce eggs. Eggs are deposited in the water along with bird faeces. Here, they hatch to release coracidia, a free-swimming larval stage that is ingested by cyclopoid copepods, the first intermediate hosts. In the copepod, coracidia migrate to the haemocoel and develop into procercoide. Sticklebacks become infected when they eat an infected copepod. In the stickleback, infective procercoide penetrate the intestine wall and migrate to the body cavity, where they develop into plerocercoids. Plerocercoids remain in the body cavity until the fish is eaten by a bird (e.g. a heron). In the bird gut, plerocercoids develop into adult worms, thus completing the life cycle (Barber & Scharsack, 2010). *S. solidus* infection in sticklebacks can be long-lived. Accordingly, plerocercoids can attain a large size in the body cavity and can account for a large proportion of stickleback body weight.

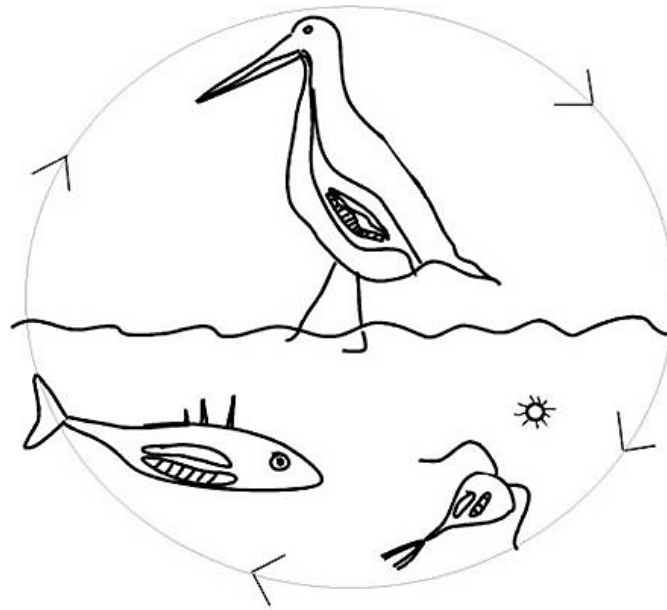


Fig. 2.5. The life cycle of *Schistocephalus solidus*. Figure from Milinski (2006).

CHAPTER 3: THE RELATIVE CONTRIBUTION OF SPATIAL AND TEMPORAL VARIATION TO MACROPARASITE COMMUNITY COMPOSITION OF STICKLEBACKS

3.1 Introduction

Parasites form an important part of the selective environment of their host organisms. Antagonistic coevolution between hosts and parasites is a key mechanism driving adaptive change in host populations (Woolhouse *et al.*, 2002; Paterson *et al.*, 2010). However, selection exerted by parasites is likely to be heterogeneous, both in space (Brockhurst *et al.*, 2004; Nuismer & Ridenhour, 2008) and time (Burdon & Thrall, 1999; Forde *et al.*, 2004; Soubeyrand *et al.*, 2009). Moreover, the most basic version of the Red Queen hypothesis, which plays a central role in our thinking about host-parasite interactions, predicts that there will be fluctuations in parasites over time (Hamilton, 1980). Therefore, the spatiotemporal structure of host-parasite interactions is crucial in determining the outcome of coevolutionary dynamics and the nature of parasite-mediated selection. Local adaptation studies have aided our understanding of host-parasite interactions in space (Lively & Dybdahl, 2000; Greischar & Koskella, 2007; Hoeksema & Forde, 2008). Likewise, temporal adaptation experiments can provide insights into the dynamics and stability of host-parasite interactions in time (Decaestecker *et al.*, 2007; Gaba & Ebert, 2009). However, spatiotemporal analyses of the parasite communities of a large number of populations of the same host species may also provide important insights, albeit indirectly, into the geographic structure and dynamics of host-parasite interactions in the wild. Indeed, a fundamental first step in assessing the impact of parasites on natural host populations is to understand their distribution (Ebert, 2005). This applies especially to vertebrate-macroparasite systems, where longitudinal data and comparative studies on host-parasite interactions in natural populations are often scarce or lacking.

An important issue in the study of parasite-mediated selection that has yet to be investigated is the relative contribution of spatial and temporal variation to parasite

distributions, and hence, parasite-mediated selection (Duffy & Forde, 2009). Parasite community ecologists have strived to identify patterns of variation in parasite community composition and structure, both in space and time (Poulin, 2007a; Kennedy, 2009). However, these two aspects are often studied in isolation (although see e.g. Behnke *et al.*, 2008). There is a need for studies that examine the generality of any patterns by looking at temporal and spatial variation in parasite community composition simultaneously across a large number of host populations. Moreover, results from such analyses are rarely interpreted in an evolutionary context that addresses the potential impact of this variation on the evolution of host populations. For instance, if host population accounts for more variation in parasite community composition than sampling time, it suggests that variation in space is more important in determining the strength of parasite-mediated selection than variation in time. This approach assumes that parasite community composition is an accurate proxy for parasite-mediated selection. Further evidence for the importance of geographical location can be obtained by examining the relationships between environmental variables and parasite distributions: if there is a strong association between parasite distributions and abiotic habitat characteristics, it indicates that the differences in parasite distributions among populations are likely to be stable in time. Habitat characteristics can influence parasite distributions for instance by determining the suitability of the environment for intermediate hosts of certain parasite species, or for the parasite species themselves (Poulin, 2007b). A large-scale comparative approach also allows one to assess whether parasite-mediated selection is acting in a consistent manner within and/or among populations (Ricklefs, 2010). For instance, if parasite distributions are sufficiently stable in time such that the relative differences among host populations do not change between sampling times, it indicates that host populations experience divergent parasite-mediated selection that is consistent in time.

Here, twelve replicate three-spined stickleback, *Gasterosteus aculeatus*, populations from North Uist, Outer Hebrides, Scotland, were sampled in two consecutive years to address the following questions: 1) is there variation in parasite communities among populations, 2) is this variation consistent across years and 3) can spatial differences in parasite community composition be explained by differences in geomorphological and physicochemical habitat characteristics? The three-spined stickleback is a useful

model species for assessing spatial and temporal variation in parasite communities for a number of reasons. It occupies a diverse array of habitats, from freshwater streams and pools to brackish/marine water bodies. Population density in these habitats is typically high, making it easy to obtain large sample sizes. Furthermore, the stickleback parasite fauna has been well-documented (Wootton, 1976; Barber, 2007). North Uist comprises an extensive network of lochs, the majority of which contain sticklebacks. Although it has yet to be assessed formally whether these populations are evolutionarily independent, the substantial morphological divergence found throughout the island (Giles, 1983b) and preliminary genetic data (S. Coyle, personal communication) suggest that some populations have been separated long enough to have diverged considerably. Therefore, the North Uist system provides a wide scope for comparative analysis.

3.2 Methods

3.2.1 Fish populations and parasite identification

Twelve stickleback populations on North Uist, Scotland, were selected for sampling to represent a range of freshwater habitats across the island (Table 3.1). The lochs were situated in a small geographical area, the furthest two lochs being 17.41km apart. Populations were sampled at the same time during the breeding season (April-May) in two consecutive years, 2007 and 2008. Fish were caught using minnow traps (Gee traps, Dynamic Aqua, Vancouver) which were set overnight and lifted the following day. A sample of 20 fish was selected haphazardly from the total number caught, although occasionally fewer than 20 fish were caught (Table 3.1). Fish were subsequently transferred to polystyrene boxes filled with lake water and provided with an air source.

Within 48 hours of capture, fish were killed, by overdose of MS222 (400 mg L⁻¹), and dissected. Standard length was measured to the nearest 0.1 mm. The external surface, gills and all organs were carefully scanned for macroparasites using a dissection microscope. The caudal, anal and dorsal fins were examined for *Gyrodactylus* and the number recorded to give an index of abundance. Presence of *Gyrodactylus* on other

parts of the body and gills were recorded in order to estimate prevalence, but were not counted. The rest of the body surface was checked for parasites under the skin. The opercular cavity and the gills on the left side were examined. Only the left eye was dissected. Intestines were stored in 70% ethanol and dissected in July-August 2007, and October-November 2008 for both years, respectively. A total of 455 fish was dissected.

Most parasites were identified to species level, using a key for parasites of freshwater fish (Bykhovskaya-Pavlovskaya *et al.*, 1964) and more current and specialist literature where necessary. Encysted trematode metacercariae found in the humour of the eye were not identified, but probably belonged to the species *Apatemon gracilis* (Blair, 1976). Here, these metacercariae are referred to as *Apatemon* sp. Likewise, encysted *Diphyllbothrium* worms could not be identified to species level and are referred to as *Diphyllbothrium* spp.

3.2.2 Statistical analysis

To identify common parasite species quantitatively, I followed the approach of MacColl (2009a). If a parasite species had an overall prevalence greater than 10%, averaged across both years, it was considered for individual statistical analysis (see below). Prevalence and abundance are defined after Bush *et al.* (1997), and refer to the percentage of hosts infected with a certain parasite species and the number of individuals of a particular parasite species on/in a host individual, respectively.

Two measures of parasite community composition at the host population level were calculated: Simpson's diversity index (1-D), which is a diversity index that takes into account the relative abundance of each species in the index (Magurran, 2003) and the percentage of fish infected with at least one parasite species. Additionally, coefficients of variation (CV) were calculated using mean abundance values for each parasite species per population, to obtain a metric indicating variability of parasite distributions across populations. Coefficients of variation are scale invariant and dimensionless and can therefore be used to compare distributions of different parasite species which have very different magnitudes of infection (Poulin, 2006).

Population	Sample size		Mean fish length (\pm S.E.)	Geographic location	Loch S.A. (km ²)	pH	Chlorophyll A conc. (μ g L ⁻¹)	DOC (mg L ⁻¹)	Ca ²⁺ conc. (ppb)
	2007	2008							
Bharpa	20	20	31.4 \pm 0.8	57°34"N; 7°17"W	0.49	6.03	6.04	4.93	165
Buaille	20	20	33.9 \pm 0.8	57°38"N; 7°11"W	0.02	6.91	3.18	3.74	274
Daimh	21	20	36.1 \pm 0.6	57°35"N; 7°12"W	0.03	6.53	-	-	240
Dubhasaraidh	12	20	36.6 \pm 1.0	57°34"N; 7°24"W	0.23	6.78	-	-	331
Hosta	20	20	41.9 \pm 1.3	57°37"N; 7°29"W	0.25	8.37	4.55	4.17	5459
Magarlan	20	20	41.5 \pm 1.0	57°36"N; 7°29"W	0.07	7.81	7.98	7.89	2321
Maighdein	20	20	32.8 \pm 0.5	57°35"N; 7°12"W	0.10	7.14	11.16	6.37	-
Mhic A'Roin	20	16	37.0 \pm 1.1	57°35"N; 7°25"W	0.08	6.63	-	-	255
Mhic Gille Bhride	11	20	39.5 \pm 0.9	57°36"N; 7°24"W	0.14	6.89	-	-	308
Moracha	20	15	32.4 \pm 0.6	57°34"N; 7°16"W	0.36	6.41	3.47	5.29	400
Scadavay	20	20	31.2 \pm 0.9	57°35"N; 7°14"W	4.88	6.12	14.87	5.00	153
Tormasad	20	20	32.1 \pm 0.7	57°33"N; 7°19"W	0.21	6.72	2.85	5.35	436

Table 3.1. The twelve freshwater lochs from North Uist sampled in the study with their geographical location, number of fish dissected for parasites per loch for both sampling years, the mean fish standard length averaged across years, loch surface area (S.A.), pH, calcium ion (Ca²⁺) concentration, Chlorophyll A concentration and dissolved organic carbon (DOC) content. Loch surface areas were calculated from 1:25,000 topographic maps using Photoshop (Adobe Systems). pH values are averages of one to four readings (depending on the loch) taken between April 2006 and April 2009. Chlorophyll A concentrations and DOC values were obtained from water samples collected in April-May 2008 by spectrophotometry and total organic carbon analyser, respectively. Calcium concentrations were obtained from water samples collected in April-May 2007 via inductively coupled plasma mass spectrometry (ICP-MS).

Parasite abundance, prevalence and species richness data, i.e. measures of parasite community composition at the level of individual hosts, were analysed using generalised linear models (GLMs). Parasite species richness was modelled with Poisson errors and a logarithm link function, whereas total parasite abundance and abundance of individual parasite species were modelled with negative binomial errors and a logarithm link function. Prevalence of individual parasite species was analysed using GLMs with a binomial error structure and a logit link function; the response variable took a value of '1' and '0' if fish were infected or uninfected, respectively. Full models were the same for all response variables and included population, year and sex as fixed effects, fish standard length as a covariate and the year \times population interaction. This term assessed whether there was a change in the relative abundance, parasite prevalence and species richness of different populations across years; if this was the case, the interaction term was expected to be significant. Length \times sex and length \times population were also fitted, to examine whether the effect of length was consistent between sexes and across populations. Significance of effects was determined by sequentially dropping each term from the full model and recording the change in deviance compared to the χ^2 distribution with the corresponding number of degrees of freedom, until a minimum adequate model was specified. If main effects were marginal to interaction effects, the significance of the main effect was assessed by dropping both the main and interaction effects. All statistical analyses were performed in GenStat (release 12, VSN International Ltd., Hemel Hempstead, UK).

To assess whether differences in environmental variables could explain spatial variation in parasite distributions, four physicochemical variables (pH, calcium concentration ('Ca²⁺ conc.'), chlorophyll A concentration ('Chlor A conc.'), dissolved organic carbon ('DOC')) and one geomorphological variable (loch surface area) were regressed against 11 measures of parasite community composition at the host population level: mean parasite species richness, mean total parasite abundance, Simpson's diversity index (1-D), *Diplostomum gasterostei* abundance and prevalence, *Gyrodactylus arcuatus* abundance and prevalence, *Apatemon* sp. abundance and prevalence, and *Schistocephalus solidus* abundance and prevalence.

Chlorophyll A concentration is a measure of phytoplankton productivity specifically, and aquatic productivity generally, whereas DOC measures organic loading of a water body. The size of water bodies (Kennedy, 1978; Hartvigsen & Halvorsen, 1994), pH (Marcogliese & Cone, 1996; Goater *et al.*, 2005; Hernandez *et al.*, 2007) and DOC (King *et al.*, 2007) have previously been shown to affect parasite species richness, and prevalence and abundance of individual parasite species in aquatic environments, and may therefore also be important in shaping stickleback parasite communities on North Uist. Loch surface area is important from an epidemiological perspective: larger lakes potentially contain larger host populations which may increase transmission of parasites (Ebert *et al.*, 2001). Calcium concentration, although strongly correlated with pH, has been shown to be associated with presence of *Diplostomum* sp. (Curtis & Rau, 1980), as lakes with low calcium concentrations cannot support the snail intermediate hosts. Furthermore, calcium concentration is known to be a dominant axis of variation among North Uist lochs (Giles, 1983b). As a result, it may affect the distribution of *Diplostomum gasterostei* and *Apatemon* sp., and was thus analysed separately from pH. DOC is known to mediate the density of invertebrates in freshwater systems (Wetzel, 2001) and may therefore affect the abundance of crustaceans, such as copepods, which are intermediate hosts for a number of stickleback cestodes, including *S. solidus* and *Proteocephalus filicollis*, and may have a knock-on effect on the abundance of these parasite species. Lastly, there are good reasons to expect a positive association between aquatic productivity and parasite species richness and abundance (Esch, 1971; Poulin *et al.*, 2003), which may also be mediated indirectly via the abundance of intermediate host species (Goater *et al.*, 2005).

Parasite data were averaged over both years for the parasite community and environmental measure associations. pH values were measured using a calibrated pH meter (Multi 340i, Semat International) and are averages of one to four readings (depending on the loch) taken between April 2006 and May 2009. Calcium concentrations were obtained from water samples, collected in April-May 2007, via inductively coupled plasma mass spectrometry (ICP-MS). The value is the average of five runs. Calcium concentrations were only available for 11 lochs. Chlorophyll A concentration and DOC values were obtained from water samples collected in April-May 2008 by spectrophotometry and total organic carbon analyser respectively. Data

for these two environmental measures were only available for 8 lochs. Loch surface areas were determined from 1:25,000 topographic maps (Ordnance Survey sheet) in Adobe Photoshop (Adobe Systems, Mountain View, CA). A matrix was constructed with r^2 values from the regression analyses. For each regression, the parasite measure was the response variable and the environmental variable was the explanatory variable.

3.3 Results

3.3.1 Parasite communities

Nine macroparasite species were recorded (Table 3.2). *Tylodelphys clavata*, a diplostomid species that is morphologically similar to *Diplostomum gasterostei*, was also recorded in the eye humour and retina. However, as *T. clavata* was encountered infrequently, findings were grouped together with *D. gasterostei*. Not included are isolated findings of a number of parasite species, which were each recorded from one fish only: the trematode *Phyllodistomum folium* (urinary bladder), the cestode *Paradilepis scolecina* (mesenteries), and two unidentified nematode species (one from the body cavity and one from the intestine). Larval intestinal cestodes were also excluded from the analyses as they were found infrequently in one population only (Hosta). Although these cestodes were unidentifiable due to their small size and underdeveloped scolex, they were most likely *Proteocephalus filicollis* and *Eubothrium crassum*, as these were the only intestinal cestodes present in Hosta (Table 3.2).

3.3.2 Spatiotemporal variation in parasite communities

Five parasite species were present in over 10% of all hosts examined and contributed strongly to variation in parasite community composition: the trematodes *Diplostomum gasterostei* and *Apatemon* sp., the monogenean *Gyrodactylus arcuatus*, and the cestodes *Schistocephalus solidus* and *Proteocephalus filicollis*. Abundance and prevalence data of these parasite species were analysed with separate statistical models.

At the host population level, parasite communities ranged from those comprising only two species, with over 80% of uninfected individuals in the population (Daimh) to those in which a large proportion (70%) of the population was infected with at least three parasite species (Mhic Gille Bhride) (Table 3.2). Parasite diversity (1-D) varied considerably among populations but population-level differences were correlated across years (Fig. 3.1a). Likewise, the percentage of fish infected with at least one parasite species was spatially variable but the relative differences among populations did not change much between years (Fig. 3.1b). Furthermore, all coefficients of variation (CV) on population mean abundances had values above 1, indicating variability among host populations for all nine macroparasite species (Table 3.2).

At the level of host individuals, parasite species richness varied significantly among populations and differed between years (Table 3.3). Although parasite species richness was higher in 2008 than in 2007, population explained substantially more variation in parasite species richness than year (Table 3.3). Moreover, the relative differences in parasite species richness among populations hardly changed between years, as indicated by the non-significant year \times population interaction and the strong positive correlation between parasite species richness in both years (Fig. 3.1c). Length was positively correlated with parasite species richness across populations (Table 3.3). Neither sex nor sex \times length explained significant variation in parasite species richness (Table 3.3). Total parasite abundance also varied significantly among populations, between years and as a result of fish length (Table 3.3). Like parasite species richness, population, rather than year, was the most important determinant of total parasite abundance. Total parasite abundance was higher in 2008 and was positively correlated with fish length across populations. The effect of length on parasite abundance differed significantly across males and females although there was no main effect of sex. The marginally significant year \times population term indicated that the relative differences in parasite abundance changed slightly between years (Table 3.3). Nevertheless, the correlation between total parasite abundance in both years remained tight and positive (Fig. 3.1d).

The GLMs of prevalence and abundance data of individual parasite species revealed common patterns. Population explained a significant proportion of variation in all data sets (Table 3.3). Year had a significant effect on the abundance of *G. arcuatus*, *D. gasterostei*, *Apatemon* sp. and *S. solidus* as well as on the prevalence of *G. arcuatus*, *D. gasterostei* and *S. solidus*, although to different extents (Table 3.3). Values of these individual parasite species measures, apart from *S. solidus* abundance, were higher in 2008 than in 2007. With the exception of *G. arcuatus* prevalence, population rather than year accounted for the largest proportion of variation in abundance and prevalence of individual parasites species. Abundance of *G. arcuatus*, *D. gasterostei*, *S. solidus* and *P. filicollis*, and prevalence of *G. arcuatus*, *D. gasterostei* and *Apatemon* sp. also varied significantly as a result of fish length. Excluding *P. filicollis* and *S. solidus*, parasite abundance was positively correlated with fish length. Likewise, prevalence of individual parasite species was higher in larger fish (Table 3.3). However, the length \times population interaction was significant for *D. gasterostei* abundance, *G. arcuatus* prevalence and *Apatemon* sp. prevalence, indicating that this relationship was not identical across all populations. Sex failed to explain variation in abundance and prevalence of all individual parasite species, but the length \times sex term was significant for *S. solidus* (Table 3.3).

The significance of the year \times population interaction term varied among parasite species (Table 3.3). The relative differences among populations in *D. gasterostei* abundance and prevalence, *P. filicollis* abundance and prevalence and *Apatemon* sp. prevalence changed little between years (Figs. 3.2 and 3.3). Relative differences in *G. arcuatus* abundance and prevalence among populations were not as repeatable across years (Figs. 3.2b and 3.3b). However, this result was highly dependent on two populations, Buaile and Scadavay. Buaile had a high *G. arcuatus* abundance in 2007 whereas the parasite was absent from the sample in 2008. Conversely, *G. arcuatus* was absent from Scadavay in 2007 but was found in 55% of fish examined in 2008. Removal of these two populations substantially improved the correlation between population-level means in both years, (*G. arcuatus* abundance: all populations, $r = 0.23$; without Buaile and Scadavay, $r = 0.99$; *G. arcuatus* prevalence: all populations, $r = 0.61$; without Buaile and Scadavay, $r = 0.86$). The year \times population interaction was also significant for *S. solidus* abundance and prevalence (Table 3.3). This may be

attributed to a single population, Mhic A'Roin. In 2007, *S. solidus* was found in the majority of fish from this population, while it was only recorded from one fish in 2008 (Figs. 3.2d and 3.3c). Again, removal of this population improved the fit of the correlation between data from both years (*S. solidus* abundance: all populations, $r = 0.68$; without Mhic A'Roin, $r = 0.85$; *S. solidus* prevalence: all populations, $r = 0.31$; without Mhic A'Roin, $r = 0.79$). The relative differences in *Apatemon* sp. abundance among populations also changed slightly between years, but the correlation between population-level means from both years remained strong and positive (Fig. 3.2c).

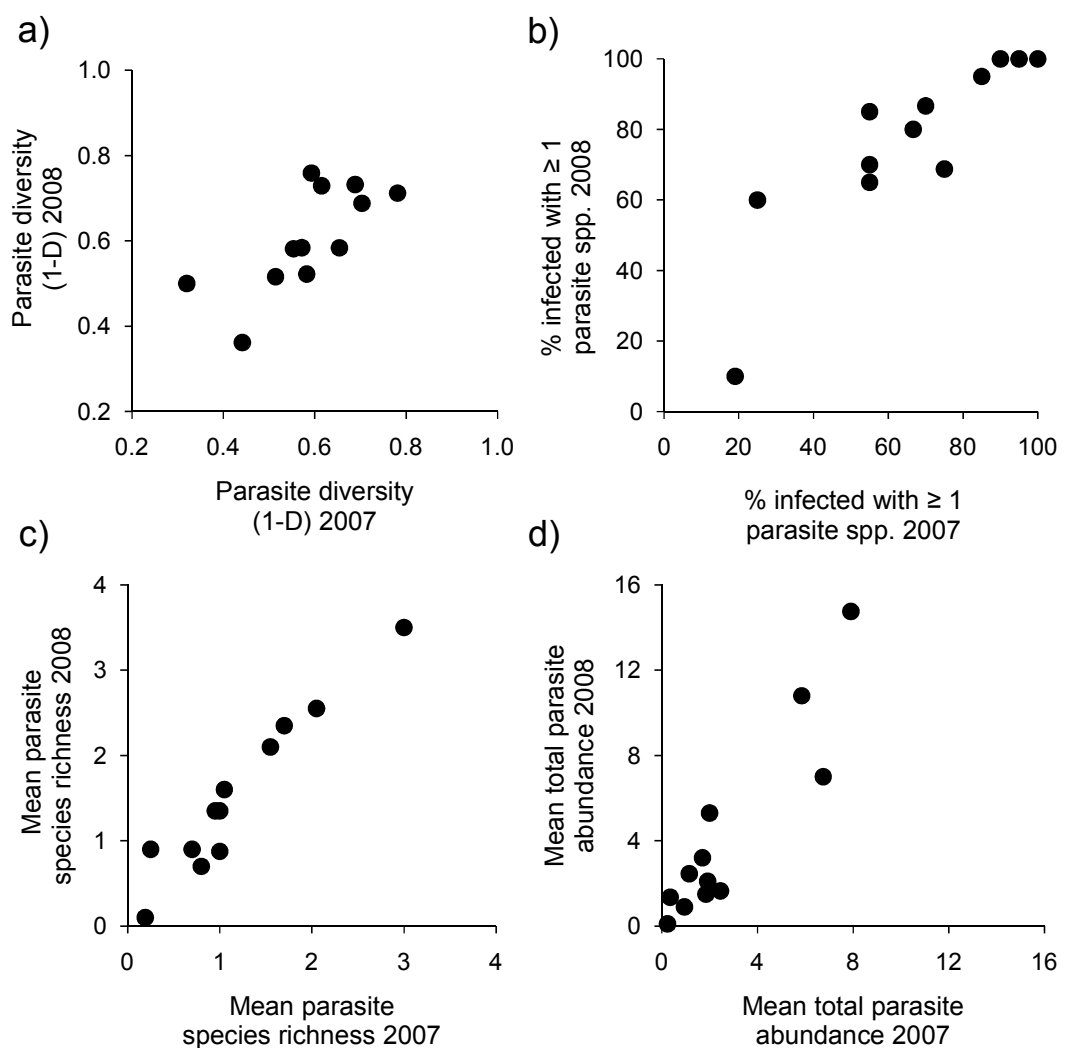


Figure 3.1 Relationship between data from 2007 and 2008 of: a) parasite diversity (1-D): $r = 0.72$; b) percentage of fish infected with at least one parasite species: $r = 0.87$; c) mean parasite species richness: $r = 0.96$, d) mean total parasite abundance: $r = 0.92$.

Taxon	Species	Year	Bharpa		Buaile		Daimh		Dubhasaraidh		Hosta		Magarlan	
			P (%)	MA	P (%)	MA	P (%)	MA	P (%)	MA	P (%)	MA	P (%)	MA
Crustacea	<i>Thersitina gasterostei</i>	2007	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	40.0	0.5
		2008	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	60.0	1.3
Monogenea	<i>Gyrodactylus arcuatus</i>	2007	0.0	0.0	35.0	3.1	0.0	0.0	16.7	0.3	45.0	1.4	40.0	0.4
		2008	0.0	0.0	0.0	0.0	0.0	0.0	60.0	0.7	90.0	2.5	35.0	0.7
Digenea	<i>Diplostomum gasterostei</i>	2007	0	0.0	80.0	2.2	4.8	0.1	58.3	1.3	20.0	0.8	40.0	0.6
		2008	5.0	0.1	90.0	2.6	5.0	0.1	60.0	1.3	35.0	0.9	55.0	2.4
	<i>Apatemon</i> sp.	2007	0.0	0.0	35.0	0.6	0.0	0.0	25.0	0.3	10.0	0.1	5.0	0.1
		2008	0.0	0.0	70.0	1.2	0.0	0.0	15.0	0.2	0.0	0.0	25.0	0.3
Cestoda	<i>Schistocephalus solidus</i>	2007	30.0	1.8	0.0	0.0	0.0	0.0	0.0	0.0	15.0	0.5	5.0	0.1
		2008	40.0	1.3	0.0	0.0	0.0	0.0	0.0	0.0	45.0	1.0	0.0	0.0
	<i>Diphyllobothrium dendriticum</i>	2007	0.0	0.0	5.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		2008	0.0	0.0	40.0	6.9	0.0	0.0	0.0	0.0	5.0	0.1	5.0	0.1
	<i>Diphyllobothrium</i> sp. (encysted)	2007	20.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	15.0	0.2	10.0	0.1
		2008	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	<i>Proteocephalus filicollis</i>	2007	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	70.0	3.4	30.0	0.3
		2008	10.0	0.2	10.0	0.2	0.0	0.0	0.0	0.0	65.0	2.3	45.0	0.7
	<i>Eubothrium crassum</i>	2007	30.0	0.5	0.0	0.0	14.3	0.2	0.0	0.0	30.0	0.6	0.0	0.0
		2008	15.0	0.2	0.0	0.0	5.0	0.1	0.0	0.0	15.0	0.4	0.0	0.0
	Parasite diversity (I-D)	2007	0.44		0.58		0.32		0.51		0.69		0.78	
		2008	0.36		0.52		0.50		0.52		0.73		0.71	
	% infected with at least one parasite spp.	2007	55.0		85.0		19.0		66.7		90.0		95.0	
		2008	85.0		95.0		10.0		80.0		100.0		100.0	

Table 3.2. See page 41 for legend

Year	Maighdein		Mhic A' Roin		Mhic Gille Bhrìde		Moracha		Scadavay		Tormasad		Overall Average		
	P (%)	MA	P (%)	MA	P (%)	MA	P (%)	MA	P (%)	MA	P (%)	MA	P (%)	MA	CV
2007	0.0	0.0	0.0	0.0	72.7	1.5	0.0	0.0	0.0	0.0	0.0	0.0	7.1	0.1	4.2
2008	0.0	0.0	0.0	0.0	65.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	10.8	0.3	3.7
2007	5.0	0.1	5.0	0.1	54.5	1.0	0.0	0.0	0.0	0.0	0.0	0.0	15.2	0.5	5.1
2008	25.0	0.3	18.8	0.4	75.0	1.7	13.3	0.2	55.0	0.8	10.0	0.1	34.2	0.6	2.0
2007	20.0	0.2	25.0	0.4	90.9	4.2	55.0	1.1	10.0	0.2	40.0	0.5	33.9	0.8	2.1
2008	30.0	0.3	12.5	0.2	100.0	9.1	80.0	1.9	15.0	0.2	45.0	1.2	44.2	1.7	1.9
2007	15.0	0.2	5.0	0.2	63.6	1.0	15.0	0.2	0.0	0.0	25.0	0.3	14.3	0.2	2.8
2008	0.0	0.0	6.3	0.1	65.0	1.4	0.0	0.0	5.0	0.5	35.0	0.5	19.0	0.3	2.8
2007	0.0	0.0	55.0	1.1	9.1	0.1	5.0	0.1	10.0	0.1	0.0	0.0	11.2	0.3	5.0
2008	0.0	0.0	6.3	0.1	15.0	0.3	33.3	0.7	10.0	0.3	5.0	0.1	12.6	0.3	3.4
2007	0.0	0.0	5.0	0.1	0.0	0.0	0.0	0.0	5.0	0.1	0.0	0.0	1.3	0.1	8.6
2008	0.0	0.0	12.5	0.2	5.0	0.1	6.7	0.1	0.0	0.0	5.0	0.1	6.5	0.6	7.0
2007	30.0	0.6	5.0	0.1	0.0	0.0	25.0	0.4	0.0	0.0	20.0	0.2	11.2	0.2	3.4
2008	10.0	0.1	0.0	0.0	5.0	0.1	13.3	0.2	5.0	0.1	5.0	0.1	3.0	0.1	5.9
2007	0.0	0.0	0.0	0.0	0.0	0.0	5.0	0.1	0.0	0.0	5.0	0.1	9.8	0.3	4.3
2008	10.0	0.2	0.0	0.0	0.0	0.0	13.3	0.1	0.0	0.0	5.0	0.1	13.4	0.3	4.3
2007	0.0	0.0	0.0	0.0	9.1	0.2	0.0	0.0	0.0	0.0	5.0	0.1	7.6	0.1	3.8
2008	10.0	0.1	31.3	0.6	20.0	0.3	0.0	0.0	0.0	0.0	20.0	0.5	9.5	0.2	3.8
2007	0.59		0.62		0.65		0.55		0.57		0.70				
2008	0.76		0.73		0.58		0.58		0.58		0.69				
2007	55.0		75.0		100.0		70.0		25.0		55.0				
2008	70.0		68.8		100.0		86.7		60.0		85.0				

Table 3.2 continued. See page 41 for legend

Response variable	Population		Year		Year × Population		Length		Sex		Length × Population		Length × Sex	
	χ^2	P	Difference ± SE	χ^2	P	χ^2	P	Estimate ± SE	P		P	P	P	P
Parasite species richness	163.3	***	0.43 ± 0.01	8.8	**	10.0	-	0.02 ± 0.01	***	-	-	-	-	-
Total parasite abundance	359.5	***	1.76 ± 0.29	33.6	***	21.1	*	0.08 ± 0.02	***	-	-	-	*	*
<i>D. gasterostei</i> prevalence	183.2	***	0.10 ± 0.01	4.9	*	6.4	-	0.11 ± 0.02	***	-	-	-	-	-
<i>D. gasterostei</i> abundance	436.8	***	0.90 ± 0.06	20.4	***	11.7	-	0.07 ± 0.04	***	-	**	-	-	-
<i>G. arcuatus</i> prevalence	69.4	***	0.19 ± 0.01	75.8	***	46.6	***	0.31 ± 0.17	***	-	*	-	-	-
<i>G. arcuatus</i> abundance	241.2	***	0.10 ± 0.04	79.0	***	71.3	***	0.07 ± 0.02	***	-	-	-	-	-
<i>Apatemon</i> sp. prevalence	138.8	***	0.05 ± 0.01	0.1	-	14.9	-	0.07 ± 0.03	**	-	*	-	-	-
<i>Apatemon</i> sp. abundance	176.3	***	0.10 ± 0.01	21.1	*	20.5	*	-	-	-	-	-	-	-
<i>S. solidus</i> prevalence	107.7	***	0.01 ± 0.01	25.4	*	24.6	*	-	-	-	-	-	-	-
<i>S. solidus</i> abundance	157.0	***	-0.02 ± 0.01	21.6	*	20.4	*	-0.05 ± 0.03	*	-	-	-	*	*
<i>P. filicollis</i> prevalence	136.6	***	0.04 ± 0.01	3.4	-	7.0	-	-	-	-	-	-	-	-
<i>P. filicollis</i> abundance	280.0	***	-0.03 ± 0.02	1.9	-	0.3	-	-0.05 ± 0.02	*	-	-	-	-	-

Table 3.3. See page 4I for legend

Table 3.2 Prevalence (P%) and mean abundance (MA) of nine macroparasite species in three-spined sticklebacks from twelve freshwater lochs in North Uist, Scotland, sampled in April-May during two consecutive years, 2007 and 2008. The mean abundance, mean prevalence and coefficients of variation (CVs) across populations are also given. Parasite diversity (Simpson's diversity index (1-D)) and the proportion of fish infected with at least one parasite species are measures of parasite community structure at the host population level. In total, 455 fish were sampled.

Table 3.3 Results from generalised linear models of parasite species richness, total parasite abundance and prevalence of *Diplostomum gasterostei*, *Apatemon* sp., *Gyrodactylus arcuatus*, *Schistocephalus solidus* and *Proteocephalus filicollis*. Parasite species richness was modelled with a Poisson error structure and a logarithm link function, total and individual parasite abundances were modelled with a negative binomial error structure and logarithm link function, whereas parasite prevalence was modelled with a binomial error structure and logit link function. Significance of effects was determined by sequentially dropping each effect from the full model and recording the change in deviance, compared to χ^2 distribution with the corresponding number of degrees of freedom. Population, year \times population and length \times population are all associated with 11 df, whereas year, length, sex and length \times sex are associated with 1 df. Probability values for the model effects were as follows: **** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$, - = $P > 0.05$. "Difference" is the difference in measures of parasite community structure between years (2008 – 2007). "Estimate" is the parameter estimate of the effect 'length' obtained from GLMs. Note that it could only be obtained when the effect of length was significant.

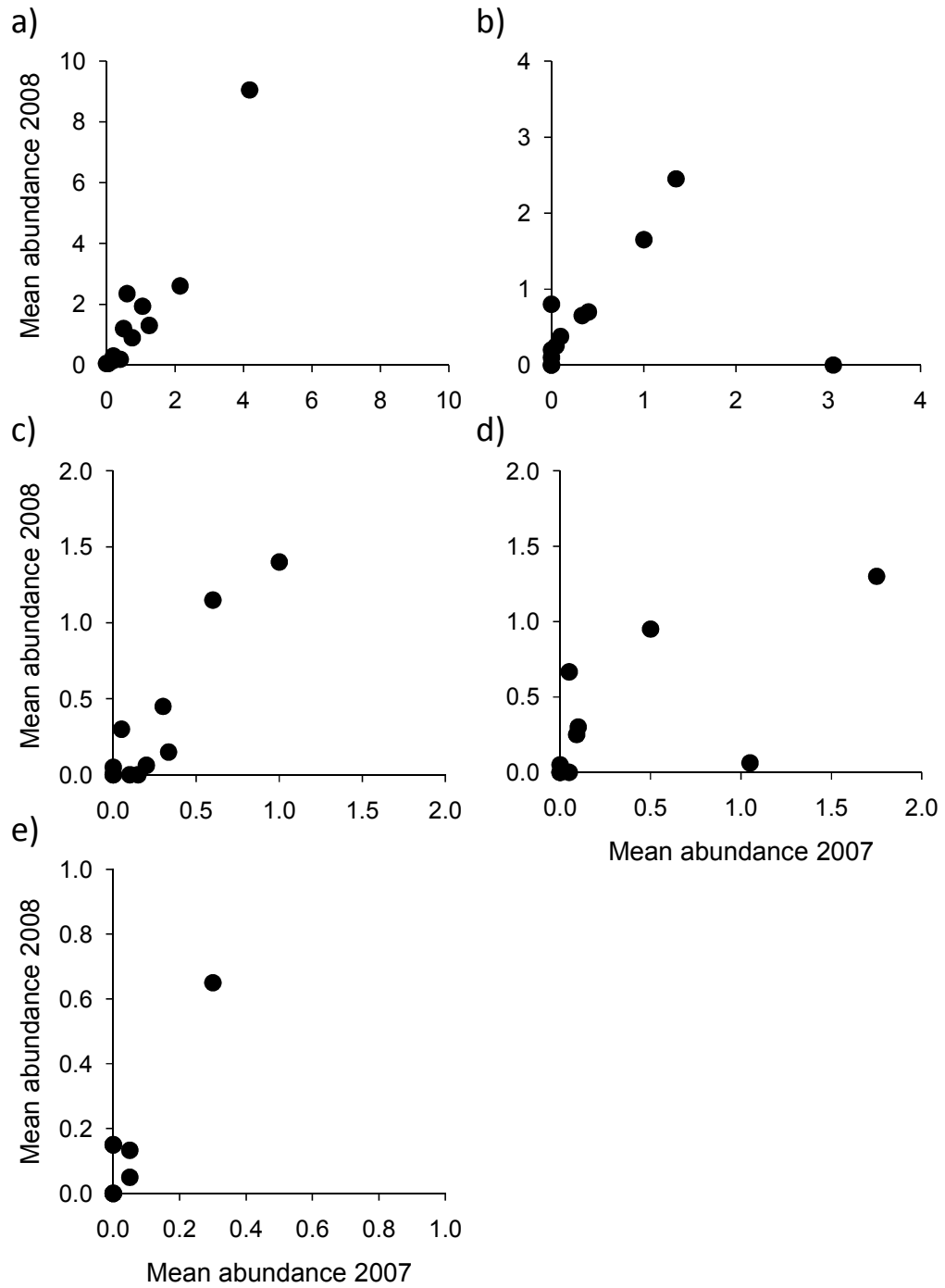


Figure 3.2 Relationship between abundance data from 2007 and 2008 of: a) *Diplostomum gasterostei*: $r = 0.96$; b) *Gyrodactylus arcuatus*: $r = 0.61$; c) *Apatemon* sp.: $r = 0.82$; d) *Schistocephalus solidus*: $r = 0.31$; e) *Proteocephalus filicollis*: $r = 0.96$.

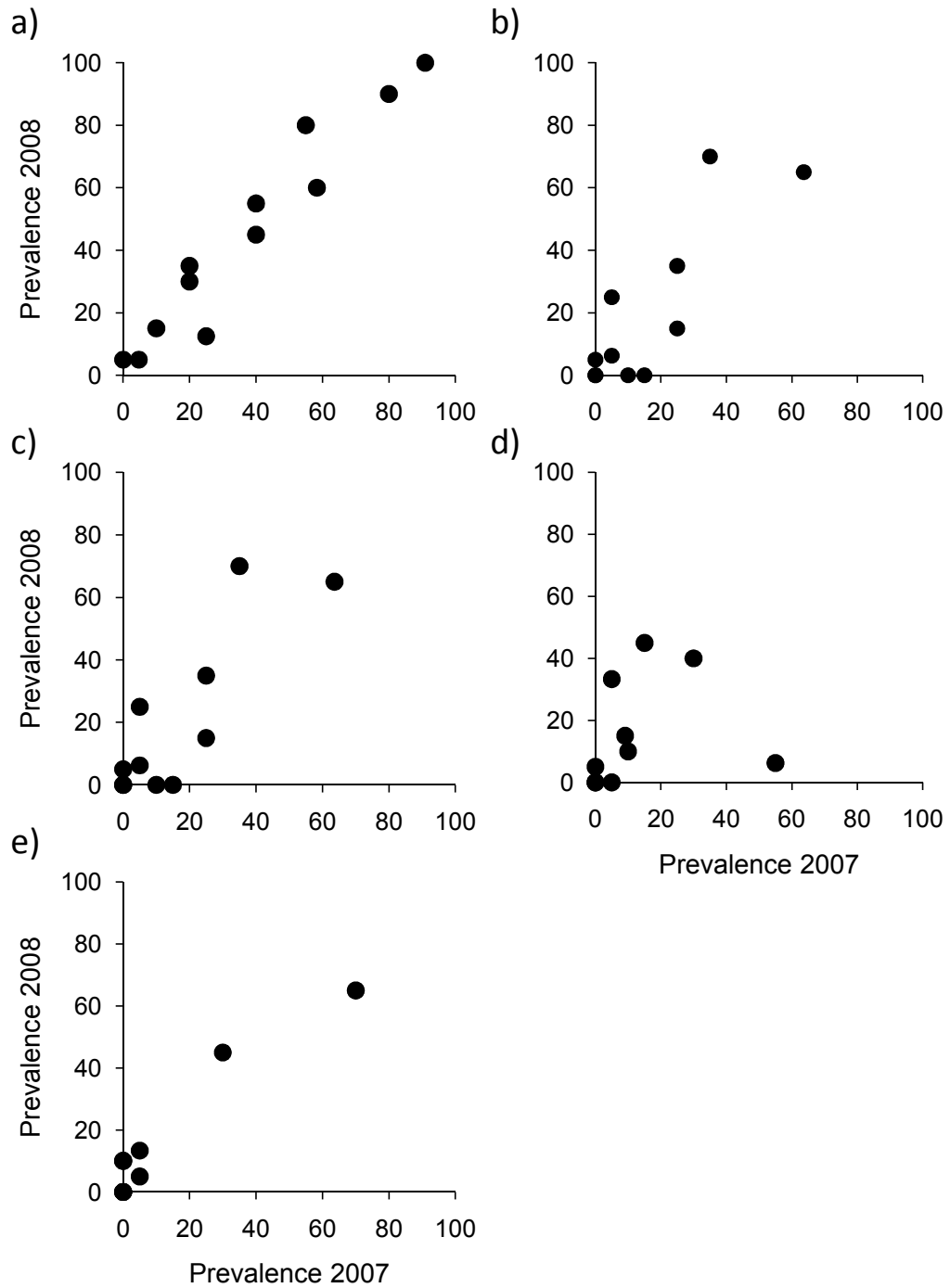


Figure 3.3 Relationship between prevalence data from 2007 and 2008 of: a) *Diplostomum gasterostei*: $r = 0.95$, $y = 2.00x - 0.22$; b) *Gyrodactylus arcuatus*: $r = 0.23$; c) *Apatemon* sp.: $r = 0.92$; d) *Schistocephalus solidus*: $r = 0.68$; e) *Proteocephalus filicollis*: $r = 0.98$.

3.3.3. Environmental variables

pH explained significant variation in parasite diversity, and *G. arcuatus* abundance and prevalence and was positively correlated with all three parasite community measures (Table 3.4, Fig. 3.4). Likewise, calcium concentration had a significant and positive association with *G. arcuatus* abundance and prevalence (Table 3.4; Fig. 3.4). No other environmental variables accounted for significant variation in other parasite community measures (Table 3.4).

Parasite community measure	pH	Loch S.A.	Ca ²⁺ conc.	Chlor. A conc.	DOC
Mean species richness	0.33	0.08	0.18	0.29	0.00
Mean total parasite abundance	0.17	0.06	0.08	0.30	0.24
Parasite diversity (1-D)	0.46	0.01	0.28	0.00	0.23
<i>D. gasterostei</i> abundance	0.02	0.04	0.01	0.34	0.01
<i>D. gasterostei</i> prevalence	0.03	0.09	0.01	0.34	0.01
<i>G. arcuatus</i> abundance	0.42	0.01	0.37	0.07	0.24
<i>G. arcuatus</i> prevalence	0.44	0.00	0.38	0.01	0.00
<i>Apatemon</i> sp. abundance	0.00	0.05	0.05	0.22	0.12
<i>Apatemon</i> sp. prevalence	0.01	0.06	0.04	0.25	0.08
<i>S. solidus</i> abundance	0.00	0.00	0.03	0.04	0.14
<i>S. solidus</i> prevalence	0.03	0.00	0.08	0.02	0.10

Table 3.4 Matrix of r^2 values from linear regressions of environmental variables (pH, loch surface area, calcium concentration, chlorophyll A concentration, DOC) against measures of parasite community structure. In each regression, the environmental measure and the parasite community measure were the explanatory variable and response variable, respectively. Parasite data were averaged over both years. Bold values indicate statistically significant ($p < 0.05$) effects of environmental measures.

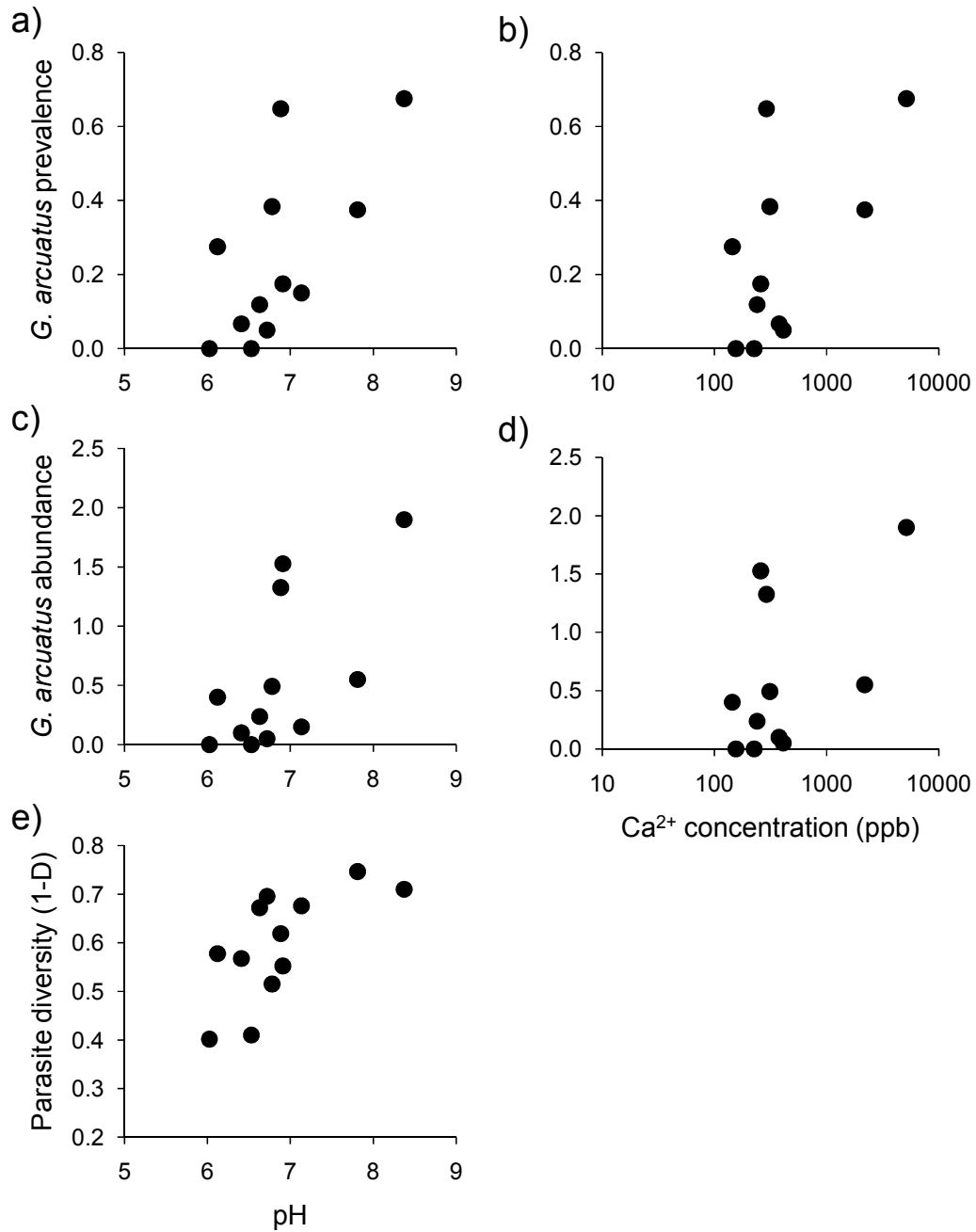


Figure 3.4 Relationship between: a) *Gyrodactylus arcuatus* prevalence and pH: $r^2 = 0.42$; b) *G. arcuatus* prevalence and calcium concentration: $r^2 = 0.37$, c) *G. arcuatus* abundance and pH: $r^2 = 0.44$; d) *G. arcuatus* abundance and calcium concentration: $r^2 = 0.38$; e) parasite diversity and pH: $r^2 = 0.46$. In all five cases, the environmental variable explained significant variation in measures of parasite community structure. pH values are averages of one to four pH readings (depending on the lochs), taken between April 2006 and May 2009. Calcium (Ca^{2+}) concentrations (parts per billion, ppb) were obtained from water samples collected in April 2007 via inductively coupled plasma mass spectrometry (ICP-MS) and were averages of 5 readings. Calcium concentrations are plotted on a log scale.

3.4 Discussion

A comparative analysis of parasite communities in twelve populations of three-spined sticklebacks was carried out to investigate the relative importance of spatial and temporal variation in determining parasite community composition. The study had three key findings. First, there was substantial variation in parasite communities among populations that were located in close geographical proximity to one another. Second, although levels of parasitism differed between years, being higher in 2008 than in 2007, population accounted for a substantially larger proportion of variation in parasite community composition of individual hosts than year. The effect of year was probably due to a warmer winter in 2007, and therefore some interaction of higher water temperatures and a more advanced breeding season, which may have led to the higher parasite prevalence and abundances in 2008. Lastly, the relative differences in parasite community composition among populations changed little across both years, a finding that is connected with the greater importance of space, rather than time, in determining parasite distribution. These patterns were observed both at the level of the whole parasite community (parasite species richness, total parasite abundance) and at the level of individual parasite species (abundance and prevalence).

These results are important from both an evolutionary and ecological perspective. It is becoming increasingly clear that parasite-mediated selection is likely to vary in space and time (Gandon *et al.*, 2008; Duffy & Forde, 2009). Although the best and most direct evidence of parasite-mediated selection in natural populations comes from tracking host and parasite genotypes in time (Dybdahl & Lively, 1998; Decaestecker *et al.*, 2007; Duncan & Little, 2007), this approach is not feasible for dissecting the relative contribution of temporal and spatial variation to parasite-mediated selection in most vertebrates-macroparasite interactions, due to the long generation times of vertebrate hosts. Using measures of parasite community composition as a proxy for the magnitude of parasite mediated selection, it may be possible, at least indirectly, to understand spatiotemporal variation in parasite-mediated selection. Following this argument, the results from this study suggest that the populations experience divergent selection that is consistent in time in the short term, and that variation in

space is more important in determining the strength of parasite-mediated selection than variation in time for sticklebacks on North Uist. From an ecological perspective, the study indicates that local host and environmental factors are crucial in shaping the composition of parasite communities (Poulin, 2007b; Kennedy, 2009; but see Thieltges *et al.*, 2009).

In comparison to stickleback populations from Germany (Kalbe *et al.*, 2002) and British Columbia (MacColl, 2009a), North Uist sticklebacks have relatively depauperate macroparasite faunas. Even in the most species-rich loch, fish with more than four parasite species were rarely encountered. The only parasites species found regularly were a monogenean (*Gyrodactylus arcuatus*), trematodes (*Diplostomum gasterostei* and *Apatemon* sp.) and cestodes (e.g. *Schistocephalus solidus* and *Proteocephalus filicollis*) and these five species largely influenced composition of parasite communities. Of the five common parasite species, *D. gasterostei*, *G. arcuatus* and *S. solidus* are particularly strong candidates for exerting parasite-mediated selection.

Diplostomum negatively impacts stickleback fitness via its effects on vision (Owen *et al.*, 1993), which consequently reduces predator avoidance (Crowden & Broom, 1980) and foraging efficiency (Seppälä *et al.*, 2004) and may lead to mortality of heavily-infected individuals in the wild (Pennycuik, 1971b). *D. gasterostei* was present in the majority of populations but prevalence and abundance varied substantially among populations. As the effect of year was marginal, relative differences in *D. gasterostei* abundance and prevalence among populations were highly repeatable across years. Therefore, selection mediated by *D. gasterostei* may vary more in space than in time. Unexpectedly, calcium concentration failed to predict spatial differences in *D. gasterostei* abundance and prevalence. It has previously been shown that calcium concentration is associated with *Diplostomum* sp. distribution (Curtis & Rau, 1980), presumably because it determines the suitability of a lake for the intermediate snail host. On North Uist, it may be the density of the snail population, rather its presence, which shapes *D. gasterostei* distribution. Parasite dispersal, mediated by birds, which serve as final hosts for *D. gasterostei*, is also known to influence trematode distribution (Marcogliese *et al.*, 2001a; Hechinger & Lafferty, 2005; Byers *et al.*, 2008).

G. arcuatus is a viviparous monogenean that lives on the gills, fins and body of sticklebacks and reproduces directly on the host. *Gyrodactylus* spp. can cause considerable parasite-induced host mortality as a result of rapid population growth (Lester & Adams, 1974). Generally, *G. arcuatus* distribution followed similar patterns to *D. gasterostei*: population explained a larger proportion of variation in *G. arcuatus* abundance and prevalence than year. As a result, the relative differences among populations changed little across years. *Gyrodactylus* sp. abundance and prevalence are known to vary seasonally (Raeymaekers *et al.*, 2008). Here, seasonality was controlled for by sampling at the same time of year, and in spite of the potential dynamism and lability of *Gyrodactylus* infection in the wild (Bakke *et al.*, 2007), this study revealed a strong degree of stability in distribution of *G. arcuatus* among populations across years. The positive relationship between pH, calcium concentration, which is closely correlated with pH, and the abundance and prevalence of *G. arcuatus* provides evidence that habitat characteristics may maintain spatial differences in *G. arcuatus* distribution. It also emphasises the importance of spatial variation as a determinant of selection mediated by *G. arcuatus*. Ectoparasites are particularly strongly influenced by their physicochemical environment (Ebert *et al.*, 2001) and are sensitive to environmental change (Khan & Thulin, 1991). Acidic conditions are known to impair survival of *Gyrodactylus* (Soleng *et al.*, 1999), which may explain why *G. arcuatus* was found at lower abundance and prevalence in acidic lochs.

S. solidus is a common body cavity cestode that is frequently cited as an agent of selection in stickleback populations due to its detrimental effects on stickleback growth (Barber *et al.*, 2008) and reproduction (Heins & Baker, 2008). Again, abundance and prevalence of *S. solidus* varied more strongly among populations than between sampling times. Therefore, the strength of *S. solidus*-mediated selection may also be determined primarily by host population rather by temporal variation in its distribution. Furthermore, with the exception of one population, the relative differences in *S. solidus* abundance and prevalence among populations were repeatable. Loch size and physicochemical variables failed to explain spatial variation in *S. solidus* distribution. Like *D. gasterostei*, *S. solidus* has a complex life cycle, being trophically transmitted from copepods to sticklebacks to birds (Barber & Scharsack, 2010). Hence, I expect similar ecological processes, such as the presence and

abundance of copepods and dispersal mediated by avian final hosts, to govern distribution of *S. solidus*.

Parasite diversity itself may act as an agent of selection (Wegner *et al.*, 2003; Bordes & Morand, 2009). Infection with multiple parasite species is the norm for many host species (Cox, 2001) and interactions between parasite species can contribute, if not govern, parasite community structure, both within a host individual and at the host population level (Pedersen & Fenton, 2007; Behnke, 2008). Therefore, the role of multiple infection as a driver of epidemiological and evolutionary processes may differ among populations as a result of variation in the composition of parasite communities (Kalbe *et al.*, 2002). Whether or not this is the case in the depauperate macroparasite communities documented here, the consideration of multiple-species infection raises an interesting question, one that has received little attention in evolutionary ecological studies of host-parasite interactions, namely: what forms a good predictor of parasite-mediated selection (Clayton *et al.*, 1992; King & Lively, 2009)? Is it a measure at the level of the entire parasite community, such as parasite species richness or parasite diversity, or is it the abundance and prevalence of individual parasite species?

Length explained significant variation in parasite species richness, total parasite abundance, and abundance and prevalence of certain parasite species. Generally, length was positively correlated with measures of parasite community structure. Although most of the stickleback populations sampled in this study are annual, some may be multi-annual (MacColl, unpublished data), and therefore differences among populations may be due to larger fish accruing more parasites over time. Equally, as the majority of populations are annual, it limits the possibility of epidemiological 'carry-over' from one year to the next. An alternative, yet compatible ecological explanation for the positive correlation is that fish that grow faster eat more infected intermediate hosts or come into contact with more free-living intermediate parasite stages. The pattern may also be the result of evolutionary processes. For example, larger fish may invest more resources in growth at the expense of investing in parasite resistance, i.e. there is a genetic trade-off between the two traits (Barber *et al.*, 2001). However, these explanations do not clarify the negative relationship between parasite abundance and length for *S. solidus* and *P. filicollis*. Instead, this suggests

parasite-induced mortality of larger individuals infected more heavily with either parasite species. Some field data of *S. solidus*-infected sticklebacks support this idea (Pennycuik, 1971a).

I found no significant difference in parasite distributions between males and females. The lack of male-biased parasitism was surprising, given that it seems to be a common feature of many vertebrate host populations (Poulin, 1996; Zuk & McKean, 1996), and that it has been demonstrated previously in three-spined stickleback populations (Reimchen & Nosil, 2001). The immunosuppressive effect of testosterone is often invoked as a proximate mechanism maintaining sex differences in parasitism (Zuk & McKean, 1996). As sticklebacks were sampled during the breeding season in both years, we might expect the immunosuppressive effects of testosterone, and therefore also male-biased parasitism, to be greatest during this period (Martin *et al.*, 2008); however, this was not the case. Dietary-based, ecological sex-biased parasitism (Reimchen & Nosil, 2001) also does not seem to take place in the stickleback populations on North Uist. Although there may be no dietary differences between the sexes within populations, differences in diet could explain variation among populations in parasite community composition. For example, evolved differences among populations in resource use, feeding preference and/or diet composition (Schluter, 1995; Knudsen *et al.*, 2006) can hinder or promote the establishment and distribution of trophically transmitted parasites (MacColl, 2009a) and influence the transmission of disease (Hall *et al.*, 2007).

This study provides a reliable basis for inferring divergent parasite-mediated selection among stickleback populations on North Uist, as the data satisfy two criteria necessary for this condition: ample geographical variation in parasites that is consistent in time (MacColl, 2009a). Divergent selection, imposed by individual parasite species or parasite communities as a whole, is likely to have consequences for the evolution of numerous host traits. Foremost among these are defence traits that confer resistance to parasites, such as components of the immune system. A specific prediction of this scenario is that adaptation to local parasite communities will drive differential and optimal investment in immune response and parasite resistance, which has been demonstrated in several different host-parasite systems (Lindström *et al.*, 2004; Whiteman *et al.*, 2006; Bryan-Walker *et al.*, 2007; Hasu *et al.*, 2009).

However, I have only provided information on spatiotemporal variation in stickleback parasite communities over a relatively short time frame. Therefore, these data do not allow me to make long-term predictions about the magnitude of parasite-mediated selection. Longitudinal data sets of fish macroparasite communities are scarce, but the few examples that do exist suggest that parasite population dynamics in the short- and long-term are not congruent (e.g. Kennedy *et al.*, 2001). To assess the repeatability of the findings presented here the same lochs should be sampled in subsequent years.

To conclude, I have shown that there is substantial variation in parasite communities of sticklebacks on North Uist and that this is accounted for largely by host population, rather than year of sampling. Furthermore, this spatial variation was repeatable across years, both at the level of the whole parasite community and at the level of individual parasite species. Several of these parasite species, especially *G. arcuatus*, *D. gasterostei* and *S. solidus* potentially exert strong selection on their hosts. Taken together, this suggests that the stickleback populations examined here experience divergent parasite-mediated selection that is not transient, at least in the short term. Moreover, it suggests that spatial, as opposed temporal, variation in the macroparasite communities of three-spined sticklebacks may be more important determining the strength of parasite-mediated selection. In the chapters that follow, I will explore the consequence of adaptation to local parasite communities for the evolution of parasite resistance and other host life history traits of North Uist stickleback populations.

CHAPTER 4: DIVERGENT RESISTANCE TO A MONOGENEAN FLATWORM AMONG STICKLEBACK POPULATIONS

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4.1 Introduction

Spatial variation in traits related to fitness is thought to be a consequence of adaptation to local ecological conditions (Schluter, 2000). Divergence in ecologically relevant traits may be driven by a number of selective factors (Rundle & Nosil, 2005). The roles of competition (Schluter, 1994; Pfennig *et al.*, 2007) and predation (Reznick & Endler, 1982; Nosil & Crespi, 2006) have been well studied in this context, but the influence of parasites on this process has been comparatively neglected (but see e.g. Buckling & Rainey, 2002b; Laine, 2009). Divergent parasite-mediated selection may affect the evolution of many host life history traits (Minchella, 1985; Fredensborg & Poulin, 2006), but defence traits are likely to be under the strongest selection, as they determine parasite resistance. All else being equal, directional selection should drive alleles coding for resistance to fixation and erode genetic variation (Mousseau & Roff, 1987; Houle, 1992). However, extensive additive genetic variation for parasite resistance in natural animal populations is commonplace (Henter & Via, 1995; Ebert *et al.*, 1998; Jackson & Tinsley, 2005; Cory & Myers, 2009). This diversity may be maintained via several mechanisms, including: i) negative frequency-dependent selection (Carius *et al.*, 2001; Woolhouse *et al.*, 2002), ii) costs of resistance (Sheldon & Verhulst, 1996; Rigby *et al.*, 2002), iii) fluctuating selection associated with environmental heterogeneity (Blanford *et al.*, 2003; Lazzaro & Little, 2009), iv) heterozygote advantage (MacDougall-Shackleton *et al.*, 2005) and v) sexual selection (Hamilton & Zuk, 1982).

An important aspect of host-parasite interactions that could govern the evolution and maintenance of parasite resistance among populations is spatial variation in parasite distributions. The magnitude of parasite-mediated selection may be determined both by the prevalence and abundance of certain parasite species. There are two possible outcomes of divergent parasite-mediated selection in terms of parasite resistance. On the one hand, populations at greater risk of infection should be under stronger selection to evolve resistance, which should lead to a positive correlation between natural parasite abundance and investment in parasite resistance. On the other hand, resistant populations may keep parasite prevalence and/or abundance at a low level, which should generate a negative correlation between parasite abundance and parasite resistance. The few studies that have been conducted in this context provide support for the former scenario. Bryan-Walker, Leung & Poulin (2007), for example, compared resistance to a trematode parasite, *Maritrema novaezealandensis*, in two populations of amphipods, *Paracalliope novizealandiae*, and found that the population not exposed to the parasite had significantly lower resistance in an infection experiment. Likewise, albeit at the level of parasite diversity, Corby-Harris & Promislow (2008) showed that natural bacterial species richness was positively associated with resistance to a bacterial species, *Lactococcus lactis* across 20 natural populations of *Drosophila melanogaster*. Cable & van Oosterhout (2007a) documented the opposite pattern for two Trinidadian guppy (*Poecilia reticulata*) populations infected with a laboratory strain of the monogenean flatworm *Gyrodactylus turnbulli*: the population naturally exposed to higher *Gyrodactylus* burdens (van Oosterhout *et al.*, 2006) was significantly more susceptible. However, with the exception of these and a few other studies (e.g. Little & Ebert, 2000; Kalbe & Kurtz, 2006; Hasu *et al.*, 2009), our understanding of patterns of divergence in parasite resistance among natural populations and their relationship to infection levels in the wild remains limited, especially for vertebrate-macroparasite interactions.

Artificial infection experiments involving outbred individuals from a number of natural populations differing in parasite distributions allow us to test the prediction that divergent parasite-mediated selection drives divergent parasite resistance. Here, using lab-reared individuals from five populations of three-spined sticklebacks, *Gasterosteus aculeatus*, I investigated divergence in resistance to *Gyrodactylus gasterostei*, a monogenean flatworm. Sticklebacks are a well-established model for the

study of ecologically-based divergent selection (McKinnon & Rundle, 2002). In the last 10,000 years marine sticklebacks have repeatedly invaded freshwater environments and as a result have undergone rapid evolution in a number of traits. Morphological divergence stemming from these invasion events has been particularly well characterised (Colosimo *et al.*, 2005), but considerable variation in life history (Baker *et al.*, 2008) and behavioural (Boughman, 2001) traits among populations has also been documented. There is a growing interest in understanding how host-parasite interactions fit into this diversification context (MacColl, 2009b; Matthews *et al.*, 2010a). Sticklebacks have a diverse and well-documented parasite fauna (Wootton, 1976; Barber, 2007) and therefore constitute a model species for investigating divergent parasite-mediated selection.

Gyrodactylids form a dominant component of many stickleback parasite communities (e.g. Kalbe *et al.*, 2002, MacColl 2009b). *Gyrodactylus* spp. are viviparous and lack a specific transmission stage. As worms reproduce directly on the host and have short generation times (2-4 days; Bakke *et al.*, 2007), population growth can be exponential, often leading to host mortality. Furthermore, *Gyrodactylus* is known to affect mate-choice and courtship behaviours (Houde & Torio, 1992; Lopez, 1998). Given their detrimental effects on host fitness, gyrodactylids are likely to exert strong selection on their hosts. Most of our knowledge of gyrodactylid-host interactions comes from the *Gyrodactylus salaris*-salmon (*Salmo salar*) and the *Gyrodactylus turnbulli*-guppy (*Poecilia reticulata*) systems. In both cases, there is evidence for geographic variation in resistance to *Gyrodactylus*. For example, van Oosterhout, Harris & Cable (2003) and Cable & van Oosterhout (2007b) showed that resistance to *G. turnbulli* varied among two populations of Trinidadian guppies. Likewise, different genetic stocks of Atlantic salmon differ in their ability to resist *G. salaris* (Bakke *et al.*, 1990; Dalgaard *et al.*, 2003). However, pathogenicity may vary widely among gyrodactylid species (Bakke *et al.*, 2007) and the extent to which different species drive divergence in parasite resistance remains unknown. Although we have some knowledge of the biology (Glaser, 1974; Harris, 1982) and population dynamics (Raeymaekers *et al.*, 2008) of *G. gasterostei*, this study sought to shed light on pathogenicity and infection dynamics of *G. gasterostei*, and spatial variation in the host response to this parasite species.

The main objectives of the study were fourfold: first, to look for differences in natural *Gyrodactylus* abundance among the five stickleback populations; second, to examine variation in resistance to *G. gasterostei* among these populations by carrying out an artificial infection experiment; third, to determine whether this variation was related to natural infection level, and fourth, to look for an association between *G. gasterostei* infection and stickleback growth. This last question was of interest because it has recently been suggested that *Gyrodactylus* may affect host life history evolution in terms of growth, at least in guppies (Cable & van Oosterhout, 2007a). Therefore, I was motivated to examine the relationship between parasite resistance and growth.

Additionally, a second infection experiment was carried out to investigate host preference of *G. gasterostei*. If *G. gasterostei* exhibits a behavioural preference for particular host genotypes (i.e. populations), then differences in parasite resistance among populations observed in the first infection experiment may also be due to population differences in host suitability. Behavioural preference relates directly to host specificity, a term that is often coined in the *Gyrodactylus* literature (Bakke *et al.*, 2002; 2007). However, it is important to distinguish between host specificity (the ability of a parasite species to infect one or a limited number of host species or populations of the same host species) and parasite resistance since they reflect a parasite trait and a host trait, respectively. The experiment employed a “two-choice” design, in which two naïve fish, one from the most resistant population and one from the most susceptible population (as determined from the large-scale infection experiment), were simultaneously exposed to an infected donor fish. To avoid confusion between the two infection experiments, the five-population comparison will hereafter be referred to as the large-scale infection experiment, whereas the behavioural preference experiment will be referred to as the two-choice experiment.

It must be emphasised that *Gyrodactylus arcuatus*, not *G. gasterostei*, is the native *Gyrodactylus* species in North Uist stickleback populations. A different *Gyrodactylus* species was chosen for the infection experiments because it removed the possibility of close coevolution between host and parasite populations. However, because there may be overlap in the host response to different *Gyrodactylus* species (Buchmann & Lindenstrøm, 2002), use of *G. gasterostei* still allowed me to make inferences about the evolution of resistance to *Gyrodactylus*. Overall, I found substantial differences in

G. gasterostei resistance among populations which were partially correlated with natural *G. arcuatus* abundance.

4.2 Methods

4.2.1 Study populations and parasites

Fish were collected from five geographically isolated lochs on North Uist, Outer Hebrides, Scotland during May 2008, using minnow traps (Gee traps, Dynamic Aqua, Vancouver). These lochs were chosen specifically to represent a range of natural infection levels (Table 4.1). Abundance and prevalence of *G. arcuatus*, the native *Gyrodactylus* species on North Uist, were estimated by sampling approximately 10-20 fish per loch in May 2008. For each population, F_1 offspring used in infection experiments were obtained by making 8 unrelated full-sib crosses (families) from wild-caught fish. To make a cross, eggs were stripped from a gravid female and placed into a petri dish containing a small volume of 1‰ salt solution. Males were killed, by overdose of anaesthetic (400 mg L⁻¹ MS222), and were dissected to remove testes. Fine forceps were used to tease apart testes and release sperm, which was gently mixed with the eggs (Barber & Arnott, 2000). Two to three hours later, fertilisation was confirmed by low-power microscopy, and testes were removed from the fertilised clutches. Fertilised eggs were transferred to a falcon tube containing 50 mL of 1 ‰ salt solution. Eggs were then transported on ice to aquaria at the University of Nottingham, where they were placed in a plastic cup with a mesh screen on the bottom, suspended in a well-aerated tank containing dechlorinated water (Marchinko & Schluter, 2007). Water was treated with Methylene blue to reduce the possibility of fungal infection. After 10 days, egg cups were transferred to individual half-tank partitions of 100L tanks and the eggs were allowed to hatch. Following hatching, full-sib families were thinned to groups of 15. Clutches from each population were distributed haphazardly between tanks across the temperature-controlled room (13.5°C ± 1°C). Fry were fed with infusoria (*Colpidium* spp.) for the first five days, then daily with brine shrimp (*Artemia salina*) naupliae until 64 days post-hatching. Thereafter, fish received chironomid larvae ('bloodworm'; defrosted from frozen)

daily. Fish were maintained at a daylight regime mimicking the natural photoperiod on North Uist.

Gyrodactylus gasterostei is a common fin parasite (Glaser, 1974; Harris, 1985) of three-spined sticklebacks. Sticklebacks infected with *G. gasterostei* were caught by hand net from a stream in Clifton, Nottingham (52°55"N; 1°10"W) at the end of February, two weeks prior to the start of the experimental infection. Gyrodactylids were identified as *G. gasterostei* by confirming the absence of excretory bladders, a defining anatomical feature of this *Gyrodactylus* species, under a dissecting microscope. Donor fish were housed in groups of 16-20 to encourage growth of parasite populations.

Population	<i>G. arcuatus</i> abundance	<i>G. arcuatus</i> prevalence	N	Geographic location
Chadha Ruaidh	0	0 (0.0, 15.9)	21	57°35"N; 7°11"W
Hosta	2.45 ± 0.51	90.0 (68.0, 98.2)	20	57°37"N; 7°29"W
Lochmaddy	12.63 ± 2.42	100.0 (86.1, 100.0)	24	57°36"N; 7°10"W
Reivil	6.27 ± 3.25	90.9 (59.6, 99.5)	11	57°36"N; 7°30"W
Tormasad	0.10 ± 0.07	10.0 (1.8, 32.0)	20	57°33"N; 7°19"W

Table 4.1 The five stickleback populations from North Uist, Scotland used in the study. Data on *Gyrodactylus arcuatus* prevalence and abundance were obtained in May 2008 by sampling approximately 20 sticklebacks from each loch. Abundance on individual fish was quantified by counting the number of worms on the caudal, anal and dorsal fins. Abundance values are given with standard error of the mean, whereas prevalence values are given with 95% confidence intervals.

4.2.2 Design of the large-scale infection experiment

In total, 150 ten-month old sticklebacks were exposed to *G. gasterostei*: 30 fish per population balanced for logistic purposes across two blocks. For each population, fish from all 8 full-sib families were included, with the exception of Hosta, for which only 7 families were available. Families were also balanced across both blocks, such that at least one fish per family was included in both blocks. Fish were housed individually in

a three-litre tank containing one litre of dechlorinated tap water. This enabled the infection profile of each fish to be monitored accurately. Water temperature was maintained at 12°C ($\pm 0.5^\circ\text{C}$), a well-established temperature for carrying out gyrodactylid infections (Bakke et al., 2002). Populations were distributed equally across the room to balance any microclimatic effects on parasite population growth.

4.2.3 Infection protocol

Donor fish, selected randomly from the infected fish population, were killed with an overdose of MS222 (400 mg L⁻¹) and placed in a Petri dish containing a small amount of dechlorinated water. *Gyrodactylus* worms were removed using insect pins. Prior to infection, the standard length of each recipient fish was measured to the nearest 0.1mm. Infection of naive, lab-bred fish was achieved by holding the caudal fin of a lightly anaesthetised (MS222, 100 mg L⁻¹) experimental fish near two previously isolated *Gyrodactylus* until the worms moved onto the fin. Generally, this process was extremely rapid: most worms transferred within 5 to 10 seconds. On the day following infection, each fish was scanned carefully using a binocular microscope to determine establishment success. If a fish had lost both parasites, it was immediately re-infected with two new worms from another randomly selected donor fish. Fish from different populations were infected in a sequential order such that all populations were exposed as uniformly as possible to worms from each donor fish, minimising any variation in infection response profiles due to worm origin.

Starting on day 4, the number of parasites on each fish was counted every four days until the end of the experimental period (day 62), by which time all but 10 fish had lost the infection. Monitoring parasite levels involved careful scanning of the caudal, anal, dorsal and pectoral fins as well as the dorsal spines, pelvic spines and girdle, caudal peduncle, flanks and head. Both stereomicroscopic and sub-stage illumination were used to accurately determine the number of *Gyrodactylus*. Prior to scanning, fish were lightly anaesthetised. On day 62, fish were killed by overdose of MS222, measured as before (standard length to the nearest 0.1 mm) and sexed by dissection. Water was changed every four days and fish were fed to satiation once per day with bloodworm, defrosted from frozen. Throughout the infection experiment, if fish were in a state of

poor health, here defined as the cessation of feeding and reduction in movement, they were euthanised by overdose of MS222 and destruction of the brain, as required by conditions of the experimental licence. For the purposes of the experiment, this was defined as mortality. By the end of the 62-day period, 13 fish had been euthanised, mostly because of infection with the secondary, opportunistic fungus of the genus *Saprolegnia*, which normally results in death of the fish once the fungus becomes visible (Pickering & Willoughby, 1982).

4.2.4 Two-choice experiment

A two-choice experiment was conducted in October 2009, approximately 7 months after the large-scale infection experiment, to assess behavioural preference of *G. gasterostei*. In the experiment, two naive fish, one from the most susceptible population (Chadha Ruaidh) and one from the most resistant population (Tormasad), were simultaneously exposed to an infected donor fish. Infected donor fish were obtained by artificially infecting naive fish from another North Uist loch (Reivil) with two *G. gasterostei* from wild caught sticklebacks from Clifton, Nottingham in September 2009. The infection protocol was identical to the one used in the large-scale infection experiment. The two-choice experiment was then conducted 23 days after infection of the Reivil donor fish. By this stage, parasite populations on the donor fish approach their peak (Fig. 4.1), thereby encouraging transmission of worms. Recipient Chadha Ruaidh and Tormasad fish, and donor Reivil fish originated from four full-sib families different to those used in the large-scale infection experiment. Prior to exposure of recipient fish, their standard length was measured to the nearest 0.1mm. One recipient fish from Chadha Ruaidh and Tormasad were size matched and randomly assigned to donor fish. Additionally, before recipient fish were transferred, parasite burdens of donor fish were quantified and the water volume in the tank was increased from one to two litres. This restricted volume ensured a similar level of contact, and hence transmission, between donor fish and both recipient fish. Then, 24 hours after exposure, parasite burdens on recipient and donor fish were quantified. In favourable conditions, gyrodactylid worms can give birth within a day of having been born (Cable & van Oosterhout, 2007a). Therefore, the short exposure period ensured that the number of worms transmitted from donor to recipient hosts could be

estimated accurately with minimal contribution from worms born on the recipient fish. Twenty replicates were performed.

4.2.5 Statistical analysis

All statistical analyses were conducted in GenStat (release 12; VSN International Ltd., Hemel Hempstead, U.K.). For all models, parameter estimates are given for significant effects only. Natural abundance of *G. arcuatus* was modelled using a generalised linear model (GLM) with a negative binomial error distribution and log link function. Population was the only fixed effect in this model.

The *Gyrodactylus* infection process is highly dynamic and is characterised by a standard sequence of events: the parasite population grows, reaches a peak, then starts to decline and is eventually cleared by the host (Fig. 4.1). Where possible, five response variables were extracted from the infection response profiles of individual fish in the large-scale infection experiment. ‘Peak’ was the parasite abundance at the peak of the infection. ‘AUC’ was the sum of worm abundances counted throughout the experiment, a measure equivalent to total worm burden over the infection time course. ‘Average r to peak’, the average daily growth rate of the parasite population, was calculated as: $r = [\ln(N_{\text{peak}}) - \ln(N_0)]/t$, where N_{peak} = ‘peak’, N_0 is number of parasites on the host at the start of the infection (two worms), and t is the day at which the peak was reached (i.e. ‘time until peak’). ‘Time lost post-peak’ was the time until clearance following the peak. For those fish that had not lost the infection by day 62, this day was noted as the day of clearance.

To reduce the number of response variables to two explanatory variables that captured the most variation in infection response profiles, a principal components analysis (PCA) was conducted on the five response variables. The resulting scores of principal component 1 (PC1) and principal component 2 (PC2) were used to make inferences about variation in resistance to *G. gasterostei*. Although this approach obscures interesting and subtle differences in temporal aspects of each infection response profile, single measures of resistance provide a more rigorous basis for statistical analysis. Due to the strong effect of AUC and peak on PC1 scores (see Results), models with these two response variable generated qualitatively similar

results to those using PC1. Nevertheless, I chose to use PC1 because it gave the best objective descriptor of the dynamics of the infection. It was not possible to calculate PC scores for those fish (13) missing values of any of the response variables due to mortality.

GLMs with a binomial distribution and a logit link function were used to model parasite establishment and host mortality data. The binary response variable, 'established?' took a value of 1 if the two parasites remained on the fish the first day following infection and 0 if they did not. Likewise, the response variable 'died?' took a value of 1 if fish died during the experimental period and 0 if fish survived. Full models included block, sex, and population as fixed effects and initial length as a covariate. For the mortality model, an additional effect, 'daily r', describing average daily growth rate of the parasite population up until the point of death or until the peak of the infection, was included to examine whether mortality was associated with infection levels. Similarly, a daily $r \times$ population effect was fitted to assess whether this relationship was consistent across populations. Significance of fixed effects was assessed by comparing the change in deviance upon dropping the effect to a Chi-square distribution with the appropriate degrees of freedom. Additionally, the relationship between mortality and *G. gasterostei* resistance at the population level was examined by a Pearson correlation.

Variation in infection response profiles was analysed using a general linear model, with PC1 or PC2 score as the response variable. To achieve normality, PC1 and PC2 scores were log and square-root transformed, respectively. The model comprised the fixed effects block, sex, population, the covariate initial length and the initial length \times population interaction. Family nested within population (population \times family) was initially included as a random effect; however, as the effect was non-significant for both PC1 and PC2 models (likelihood ratio (LR) test based on comparing the deviance of the reduced model without the random interaction term and the deviance of the full model (Galwey, 2006) – PC1: $\chi^2_1 = 0.78$, $P = 0.189$, variance component estimate \pm S.E. = 0.0030 ± 0.0038 ; PC2: $\chi^2_1 = 0.37$, $P = 0.272$, variance component estimate \pm S.E. = 0 ± 0), the model reverted to a GLM. Stepwise regression was used to construct a minimal adequate model by sequentially dropping non-significant fixed effects (sensu

Crawley, 2007). If main effects were marginal to interaction effects, significance of main effects was tested in the presence of interactions. *Post-hoc* contrasts on log-transformed PC1 scores were used to examine differences among populations in more detail. The correlation between mean population (log-transformed) PC1 scores and mean natural abundance of *G. arcuatus* was tested using a Pearson correlation and a two-tailed significance test.

To examine if there was variation in fish growth rate, and a relationship between parasite resistance and fish growth, a linear mixed model was used. Specific growth rate (SGR; *sensu* Barber, 2005) the average daily percentage increase in fish length, was calculated using the equation: $SGR = 100 * [\ln(L_{62}) - \ln(L_0)] / 62$, where L_0 and L_{62} denote the length measured before, and at the end of, the experimental infection, respectively. SGR was square-root transformed to achieve normality. The full model consisted of family nested within population as a random effect and block, sex, initial length, population, PC1 score (untransformed) and population \times PC1 as fixed effects. Significance of the random effect was assessed using a LR test. As above, non-significant fixed effects were dropped from the full model to generate a minimum adequate model. Significance of fixed effects was determined by Wald F tests.

A GLM was used to analyse data from the two-choice experiment. The response variable 'recipient burden' was modelled with a Poisson distribution and a logarithm link function. The model included the fixed effects recipient length, recipient population, donor length, donor burden and the donor length \times donor burden and recipient population \times donor burden interaction effects. These terms test whether longer fish with bigger parasite burdens were disproportionately more likely to transmit parasites to recipient fish, and whether the relationship between donor burden and recipient burden varied among populations, respectively.

4.3 Results

4.3.1 Natural *G. arcuatus* infection

Natural *G. arcuatus* abundance varied significantly among populations ($\chi^2_4 = 54.98$, $P < 0.001$; Table 4.1). *G. arcuatus* appeared to be absent from one population, Chadha Ruaidh.

4.3.2 Parasite establishment and host mortality in the large-scale infection experiment

Eight fish lost their infection within 24 hours; however, all 8 fish were successfully re-infected the following day. *G. gasterostei* establishment did not vary significantly among populations ($\chi^2_4 = 5.00$, $P = 0.288$). It also did not differ significantly between males and females ($\chi^2_1 = 0.01$, $P = 0.931$), blocks ($\chi^2_1 = 0.00$, $P = 0.991$) or as a result of variation in fish length ($\chi^2_1 = 0.00$, $P = 0.956$). Although there was some small-scale variation in mortality among populations (number of deaths per population: Chadha Ruaidh, 5, Hosta, 2, Lochmaddy, 1, Reivil, 3, Tormasad, 2), the effect of population was not significant ($\chi^2_4 = 3.99$, $P = 0.407$). Likewise, mortality was not dependent on the average daily growth rate of the parasite population (daily r : $\chi^2_1 = 0.11$, $P = 0.745$) or its interaction with population (daily $r \times$ population: $\chi^2_4 = 3.82$, $P = 0.431$). Sex ($\chi^2_1 = 0.32$, $P = 0.574$), length ($\chi^2_1 = 1.77$, $P = 0.184$) and block ($\chi^2_1 = 2.58$, $P = 0.108$) also failed to explain significant variation in host mortality. The relationship between mortality and population mean PC1 score was positive but not significant ($r = 0.687$, $P = 0.200$).

4.3.3 Resistance to *G. gasterostei*

Principal component 1 (PC1) explained 46.92% of the variation in infection response profiles and was determined mainly by the peak and AUC (Table 4.2). These two variables were strongly positively correlated ($r = 0.94$; Table 4.3). PC2 accounted for 28.87% of variation and was influenced largely by average r to peak and time until peak (Table 4.2), which were negatively correlated ($r = -0.41$; Table 4.3).

Response variable	PC1 loading	PC2 loading
AUC	0.638	0.004
Peak	0.630	0.027
Average <i>r</i> to peak	0.229	0.704
Time until peak	0.348	-0.647
Time lost post-peak	0.152	0.292

Table 4.2 Loadings from a principal components analysis (PCA) of the five response variables, extracted from the infection response profiles of individual fish. PC1 and PC2 accounted for 46.92% and 28.87% of variation in infection response profiles, respectively.

	AUC	Peak	Average <i>r</i> to peak	Time until peak	Time lost post-peak
AUC					
Peak	0.94				
Average <i>r</i> to peak	0.31	0.37			
Time until peak	0.47	0.43	-0.41		
Time lost post-peak	0.21	0.09	0.13	-0.05	

Table 4.3 A matrix of correlation coefficients between the five response variables included in the principal components analysis.

There was no significant difference in PC1 scores between the two experimental blocks, and therefore results from both blocks were pooled (Table 4.4). Mean infection response profiles were markedly different for each population (Fig. 4.1) and PC1 scores varied significantly among populations (Table 4.4). Chadha Ruaidh, the only population not naturally exposed to *G. arcuatus*, had a significantly higher PC1 score than Reivil, Tormasad and Lochmaddy (contrast: $F_{1,122} = 20.04$, $P < 0.001$). Furthermore, Tormasad had a significantly lower PC1 score than any other population (contrast: $F_{1,122} = 27.55$, $P < 0.001$). There was no significant difference in PC1 score between Chadha Ruaidh and Hosta (contrast: $F_{1,122} = 0.36$, $P = 0.552$). Initial length was negatively correlated with PC1 score across populations, but there was no significant difference in PC1 score between males and females (Table 4.4). Mean population PC1

score was weakly negatively correlated with natural *G. arcuatus* abundance rank score, although this relationship was not significant ($r = -0.23$, $P = 0.701$; Fig. 4.2). PC2 scores were significantly affected by block, being marginally higher in Block 2 than Block 1, but not by sex, population, initial length and initial length \times population (Table 4.4).

Response variable	Fixed effect	F	df	P	Estimate \pm S.E.
Log (PC1)	Initial length	6.08	1, 122	0.015	-0.011 \pm 0.005
	Population	9.57	4, 122	<0.001	
	Chadha Ruaidh				0.995 \pm 0.179
	Hosta				0.960 \pm 0.194
	Lochmaddy				0.816 \pm 0.169
	Reivil				0.876 \pm 0.192
	Tormasad				0.686 \pm 0.174
	Sex	0.91	1, 121	0.341	-
	Block	0.06	1, 120	0.803	-
	Initial length \times population	0.91	4, 116	0.462	-
Sqrt (PC2)	Initial length	2.29	1, 125	0.133	-
	Population	0.35	4, 121	0.843	-
	Sex	0.01	1, 120	0.932	-
	Block	5.64	1, 126	0.019	
	Block 1				1.621 \pm 0.044
	Block 2				1.767 \pm 0.043
	Initial length \times Population	1.26	4, 116	0.292	-

Table 4.4 Results from general linear models of PC1 and PC2 scores, used to characterise variation in infection response profiles of individual sticklebacks.

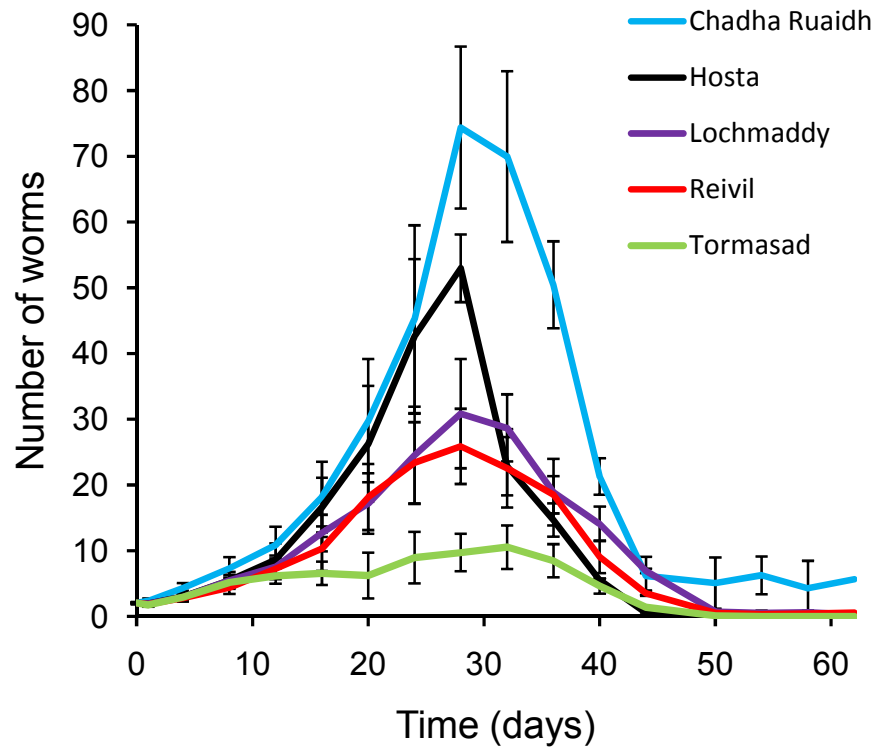


Figure 4.1 Mean infection response profile of the five stickleback populations across the 62-day experimental period. Parasite abundance of *Gyrodactylus gasterostei* on individual fish was counted every four days, following artificial infection with two *G. gasterostei* on Day 0. Data from fish that died were included until the time of death. Error bars are the standard error of the population mean at each time point.

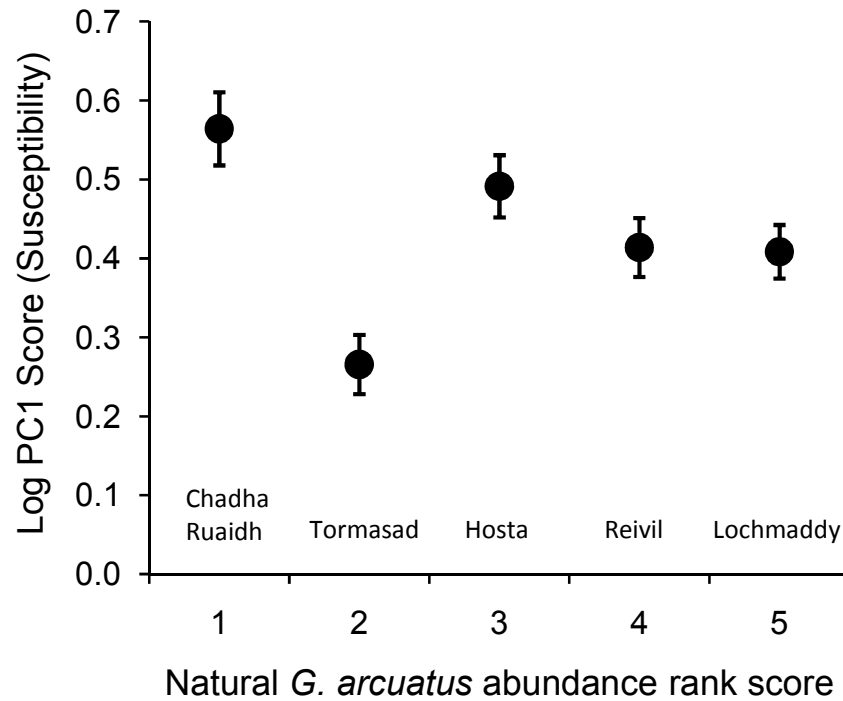


Figure 4.2 Relationship between population means of log-transformed PC1 scores and natural *G. arcuatus* abundance rank score. PC1 scores served as a proxy for resistance to *G. gasterosteii*: a high PC1 value indicates susceptibility, whereas a low PC1 value indicates resistance. Rank score increase corresponds to an increase in natural *G. arcuatus* abundance. Error bars are the standard error of the population mean. The correlation was negative and not significant ($r = -0.23$, $P = 0.701$).

4.3.4 Associations between *G. gasterostei* resistance and fish growth

Specific growth rate varied significantly among families (LR test of population \times family: $\chi^2_1 = 11.84$, $P < 0.001$; variance component estimate \pm S.E. = 0.00092 ± 0.00039). There was no significant effect of block on SGR ($F_{1,96} = 0.78$, $P = 0.379$); hence results from both blocks were pooled. PC1 score did not explain significant variation in SGR ($F_{1,103} = 0.01$, $P = 0.911$) and this relationship did not vary among populations (PC1 \times population: $F_{4,103} = 0.54$, $P = 0.708$). However, SGR was significantly affected by population ($F_{4,35} = 31.78$, $P < 0.001$), length ($F_{4,35} = 31.78$, $P < 0.001$) and sex ($F_{1,127} = 93.55$, $P < 0.001$). Length was negatively correlated with SGR (parameter estimate \pm S.E. = -0.152 ± 0.001), such that larger fish grew proportionately slower. Females grew faster than males (parameter estimate \pm S.E.: females = 0.349 ± 0.007 , males = 0.243 ± 0.008).

4.3.5 Two-choice experiment

There was no behavioural preference of *G. gasterostei* for Chadha Ruaidh or Tormasad fish, as measured by the parasite burden of recipient fish 24 hours after exposure ($\chi^2_1 = 0.23$, $P = 0.632$). Likewise, *G. gasterostei* preference was not dependent on recipient fish length ($\chi^2_1 = 0.04$, $P = 0.832$). However, donor burden was significantly positively correlated with recipient burden ($\chi^2_1 = 12.43$, $P < 0.001$; Fig. 4.3). This relationship was consistent across Chadha Ruaidh and Tormasad ($\chi^2_1 = 1.93$, $P = 0.164$). Although the effect of donor length on recipient burden was only marginally non-significant ($\chi^2_1 = 2.85$, $P = 0.058$) the interaction between donor length and donor burden was significant ($\chi^2_1 = 5.69$, $P = 0.017$).

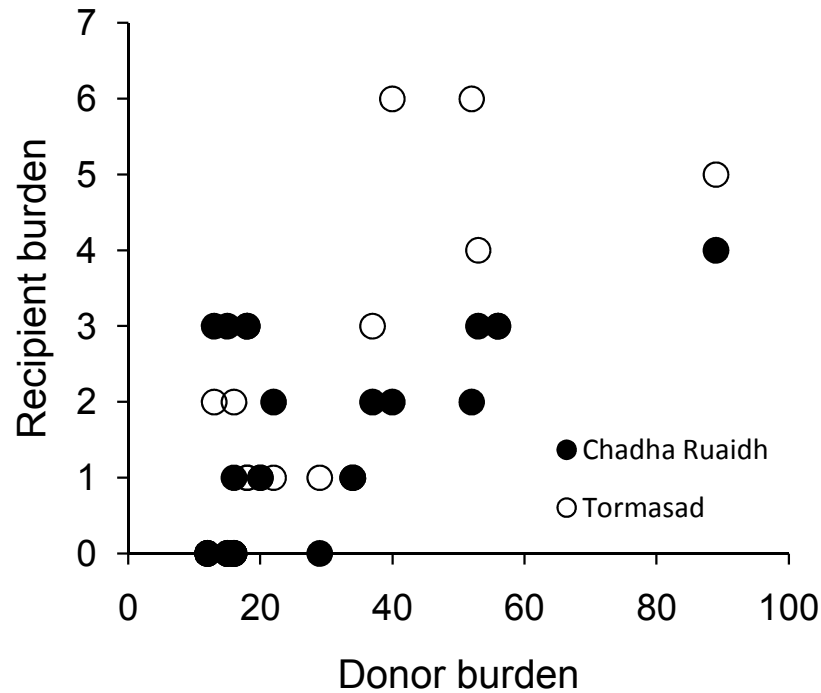


Figure 4.3 Relationship between donor burden and recipient burden in the two-choice experiment. One fish from Tormasad and Chadha Ruaidh were transferred simultaneously to a tank containing an artificially infected Reivil fish. Donor burden was quantified prior to transfer of recipient fish whereas recipient burden was quantified 24 hours after exposure (i.e. transfer). The experiment consisted of 20 replicates. Donor burden was positively correlated with recipient burden (combined: $r = 0.68$; Tormasad only: $r = 0.79$; Chadha Ruaidh only: $r = 0.55$).

4.4 Discussion

I found substantial variation in resistance to *Gyrodactylus gasterostei* among the five populations of three-spined sticklebacks. Population-level variation in resistance has been reported in other *Gyrodactylus*-host systems (Madhavi & Anderson, 1985; van Oosterhout *et al.*, 2003; Bakke *et al.*, 2004), but this is one of the few demonstrations of variation in resistance to *Gyrodactylus* among stickleback populations. A multivariate technique, used to analyse variation in infection response profiles of individual fish, revealed that resistance to *G. gasterostei* was defined largely by the ability to limit the size of the worm population, rather than by the timing of the host response to infection. Hereafter, resistance will be defined as being inversely

proportional to PC1 score, such that a low PC1 score denotes resistance and a high PC1 score denotes susceptibility.

The results of the large-scale infection experiment, utilising a common garden, suggest that *G. gasterostei* resistance has a genetic basis, echoing studies in guppies (Madhavi & Anderson, 1985; Cable & van Oosterhout, 2007a), salmon (Bakke *et al.*, 1999; Gilbey *et al.*, 2006) and topminnows (Leberg & Vrijenhoek, 1994; Hedrick *et al.*, 2001). In addition to population-level differences in resistance, fish families varied in their response to infection. This may reflect true genetic variation, although my choice of a F1 generation full-sib experimental design means I cannot exclude the contribution of maternal effects or dominance variance (Lynch & Walsh, 1998). This variation also could have been the result of host genotype x parasite genotype interactions (Lambrechts *et al.*, 2006b) generated by genetic variation in virulence in the *G. gasterostei* source population. In any case, this study provides the first line of evidence for the potential of *Gyrodactylus*-mediated selection in stickleback populations, because variation in infection response profiles was influenced by host genetics (Anderson & May, 1982; Little, 2002).

The mechanistic basis of the variation in *G. gasterostei* resistance is currently unknown. Several arms of the innate immune system have been implicated with the host response to *Gyrodactylus* (Buchmann & Lindenstrøm, 2002), especially the alternative complement pathway (Buchmann, 1998; Harris *et al.*, 1998). It would be worthwhile examining baseline complement levels of all five populations and to assess their relationship with experimental levels of resistance. This has been demonstrated for three strains of Atlantic salmon that vary greatly in resistance to *G. salaris*: higher levels of complement factor C3 in mucous corresponded to greater *G. salaris* resistance (Bakke *et al.*, 2000). Interestingly, principal component 2 (PC2) scores, which were determined mainly by the time until the peak of the infection was reached, did not vary significantly among populations, implying the existence of an immune cascade leading to clearance of the infection that is common to all populations. Therefore, quantitative, rather than qualitative differences in immune parameters may be responsible for variation in resistance among populations. Alternatively, *Gyrodactylus* resistance may be related to the density of mucous cells (Buchmann & Uldal, 1997). Although the adaptive immune system is unlikely to have a

large effect on the *Gyrodactylus* infections of naive, lab-bred fish, such as those conducted here, recent studies have found associations between major histocompatibility complex (MHC) genetic diversity and natural *Gyrodactylus* abundance (Eizaguirre *et al.*, 2009b; Fraser & Neff, 2010), suggesting that the adaptive immune response may contribute to parasite resistance in the wild.

Resistance to *G. gasterostei* was not significantly correlated with natural *G. arcuatus* abundance. However, a weak pattern emerged. Generally, fish from populations exposed to higher levels of *G. arcuatus* in the wild were more resistant to *G. gasterostei* (had a lower PC₁ score) in the large-scale infection experiment. Tormasád was the exception to this pattern, showing high resistance despite low natural burdens. Chadha Ruaidh, the only naturally uninfected population, demonstrated particularly low resistance. This supports findings from other studies that have compared evolutionary naive and exposed populations. For example, Hasu *et al.* (2009) examined variation in experimental resistance to an acanthocephalan parasite, *Acanthocephalus lucii*, among naturally exposed and unexposed isopod (*Asellus aquaticus*) populations and showed that unexposed populations were markedly more susceptible. Similarly, Kalbe and Kurtz (2006) reported that a population of three-spined sticklebacks naturally exposed to the eye fluke *Diplostomum pseudospathaceum* was significantly more resistant than a naturally unexposed population. In the absence of the parasite, there may be less selection to maintain resistance (Webster *et al.*, 2004; Lohse *et al.*, 2006; Hasu *et al.*, 2009), provided that parasite resistance is costly (Sheldon & Verhulst, 1996; Rigby *et al.*, 2002). Here, I studied a number of populations that spanned a gradient of natural infection levels, rather than considering only naturally exposed and unexposed host populations. These data suggest that differences in *Gyrodactylus*-mediated selection, as inferred from natural infection levels, may play a role in driving variation in *G. gasterostei* resistance among populations.

There are several caveats to the approach of correlating natural infection levels with experimental levels of parasite resistance, which may go some way to explaining the noisiness of the pattern. First, using current infection levels to infer historical levels of parasite-mediated selection is problematic, considering that there may be temporal variation in parasite distributions within a host population. Furthermore, the small

sample size used in this study (20 fish per population) may not capture the true extent of spatial heterogeneity natural infection levels. However, data I have collected on spatiotemporal variation in *G. arcuatus* distribution indicate that differences among populations are stable in the short term (Chapter 3), lending support to the idea that the single measure can be informative, at least for the North Uist system. Second, I chose to use a non-native *Gyrodactylus* species to investigate divergent resistance to this parasite. This decision was based on the need to remove the possibility of close coevolution between the host and parasite species. Moreover, because there may be overlap in the host response to both *Gyrodactylus* species, it allowed me to draw general conclusions about the evolution of resistance to *Gyrodactylus*. However, it is necessary to repeat the infection experiment with *G. arcuatus* to confirm that the observed differences in *G. gasterostei* resistance are relevant to infection scenarios in the wild. Thirdly, the interpretation that a positive relationship between natural infection levels and experimental resistance is evidence for divergent *Gyrodactylus*-mediated selection assumes that natural infection levels are determined mainly by the environment (exposure rate), not host genetics. If the opposite were true, I might expect to have seen a negative relationship between natural infection levels and parasite resistance. In reality, both exposure and host genetics influence parasite distribution in host populations (Scott, 1991; Grosholz, 1994; Little & Ebert, 2000; Karvonen *et al.*, 2004a). Lastly, this study included just five host populations. Inclusion of more populations could alter the sign and strength of the relationship. Nevertheless, I observed an interesting pattern that warrants further investigation. More studies are necessary to obtain a fuller understanding of how differences in parasite distribution shape investment in parasite resistance, particularly for vertebrate macroparasite interactions where such data are scarce.

Mortality in the large-scale infection experiment was low. This supports other *G. gasterostei* infection experiments (Harris, 1982) that also found low levels of mortality associated with infection. Mortality was independent of the growth rate of the parasite population, which is sometimes used as a proxy for *Gyrodactylus* pathogenicity (Bakke *et al.*, 2007). Moreover, many fish sustained large infections without any apparent pathology. By comparison, small *G. turnbulli* infections can be lethal for guppies (Scott & Anderson, 1984; Cable & van Oosterhout, 2007a). There are two possible explanations for the low mortality observed in our study. On the one

hand, low pathogenicity may be a property of the strain of *G. gasterostei* used in the infection experiment, or more generally, of *G. gasterostei* as a species. On the other hand, host tolerance to infection, defined as the ability to limit the detrimental fitness effects of the parasite (Restif & Koella, 2004; Raberg *et al.*, 2007; Raberg *et al.*, 2009), may play an important role in the *G. gasterostei*-stickleback interaction. Minimising *G. gasterostei*-associated mortality may form a separate or complementary defence mechanism to resistance to *G. gasterostei*. Furthermore, there may be variation in this strategy among populations, such that more tolerant populations are able to sustain higher parasite levels and minimise the fitness effects of *Gyrodactylus* infection relative to less tolerant populations. Interestingly, mortality did not vary significantly among populations, suggesting that there may not be genetic variation in tolerance to *G. gasterostei*. However, this idea needs to be tested explicitly in separate infection experiments.

I did not find a significant effect of *G. gasterostei* resistance on stickleback growth and this was consistent across all five populations. Unfortunately, due to the experimental design it was not possible to distinguish the effects of the host response to infection (resistance) from the effects of the infection itself. Negative effects of *Gyrodactylus* infection on host growth have been reported previously (Barker *et al.*, 2002), but there are few studies that have investigated growth effects of *Gyrodactylus* infection formally. In fish, growth rate is an important component of fitness (Schluter, 1995; Arendt, 1997). In contrast to the lack of an effect of parasite resistance on growth rate during the infection experiment, fish length, measured before the start of the infection experiment, explained significant variation in *G. gasterostei* resistance. This suggests there may be an interaction between fish growth and parasite resistance. Larger fish were more resistant to *G. gasterostei*, contradicting results from a study in guppies that found that larger guppies supported larger number of *Gyrodactylus* and were more likely to die as a result of the infection (Cable & van Oosterhout, 2007a). Larger fish are assumed to have a greater surface area that provides more niche space for parasites (Poulin, 2000). A possible explanation for the pattern observed here is that there is a positive genetic correlation between parasite resistance and host growth (Coltman *et al.*, 2001). I explore this possibility in Chapter 7. If a genetic correlation exists, selection mediated by *Gyrodactylus* may have consequences for stickleback life history evolution in terms of growth. The significant variation in

growth rate among host populations certainly suggests that there is ample inter-population variation for this life history trait. In any case, it has yet to be determined whether resistance to *Gyrodactylus* is evolutionarily or physiologically costly. Costliness of parasite resistance, like spatial variation in parasite distributions, forms a potential mechanism constraining the evolution of parasite resistance within and among host populations (Rigby *et al.*, 2002; Cotter *et al.*, 2004).

There was no behavioural preference of *G. gasterostei* for a susceptible (Chadha Ruaidh) or resistant (Tormasadh) host genotype in the two-choice experiment. The large-scale infection experiment already revealed that there were no significant differences in establishment of *G. gasterostei* among the five populations. However, because the route of infection in the large-scale experiment was highly artificial (transferring two worms onto the tail of recipient fish), any difference in establishment cannot be interpreted as a behavioural preference. The two-choice experiment simulated a more natural infection scenario by exposing recipient fish to an infected donor. Indeed, direct host-host contact is the most frequent and important mode of *Gyrodactylus* dispersal (Olstad *et al.*, 2006), and in the wild *Gyrodactylus* population dynamics are defined by the continuous transmission of worms from infected to uninfected hosts (Scott & Anderson, 1984). The lack of behavioural preference was a little surprising, given the highly significant differences in resistance between both populations. The only factor that explained significant variation in the worm burdens of recipient fish was the burden of the donor fish; as the donor burden increased, so too did the recipient burden. Increased transmission with higher worm burden appears to be a common feature of *Gyrodactylus* infection (Bakke *et al.*, 2002). The two-choice experiment confirms that variation in resistance to *G. gasterostei* observed in the large-scale infection experiment reflects variation in the host response to infection, rather than population-differences in initial suitability of fish as hosts.

In summary, I have shown that there are substantial differences in *G. gasterostei* resistance among stickleback populations on North Uist. Resistance was best defined by the ability to limit the size of the infection rather than by preventing establishment of the parasite or the timing of the host response. The population-level variation observed here most likely has a genetic basis, although the mechanism(s) conferring

resistance have yet to be explored. There was a weak positive correlation between resistance to *G. gasterostei* and natural abundance of *G. arcuatus*, suggesting that population-level differences in resistance to *Gyrodactylus* may be driven partly by divergent selection mediated by this parasite, inferred from natural infection levels of *G. arcuatus*. More generally, this study illustrates the potential of the stickleback-*Gyrodactylus* interaction as a tractable model for investigating divergent parasite-mediated selection and the evolution of parasite resistance.

CHAPTER 5: THE RELATIONSHIP BETWEEN PARASITE RESISTANCE AND THE INNATE IMMUNE RESPONSE ACROSS STICKLEBACK POPULATIONS

5.1 Introduction

Parasites are ubiquitous, as is the harm that they inflict on their hosts (Stearns & Koella, 2008). The constant threat of parasites leads us to expect the evolution of resistance where infection is associated with fitness costs. Similarly, there will be strong selection on protective mechanisms (Sheldon & Verhulst, 1996). One of the most important host defence mechanisms is the immune system (Frank, 2002; Janeway *et al.*, 2004). Given that both parasite resistance and the immune response are important contributors to host fitness (Zuk & Stoehr, 2002; Schmid-Hempel, 2003), we should expect the risk of parasitism, host resistance to parasites and the immune response to be tightly correlated. This prediction is at the heart of ecological immunology, a relatively new field that has attempted to integrate immune responses into a general life history framework (Sheldon & Verhulst, 1996; Zuk & Stoehr, 2002; Sadd & Schmid-Hempel, 2009). Indeed, many ecological immunologists that measure one or a number of immune parameters in individuals from natural populations assume that these measures accurately reflect ‘immunocompetence’, the ability of those individual to combat parasite infection (Adamo, 2004; Lee, 2006; Martin *et al.*, 2006; Sadd & Schmid-Hempel, 2009).

The problem with this ‘immunocompetence’ approach is that it decouples the immune response from its function and hence, its biological significance (Siva-Jothy, 1995; Owens & Wilson, 1999; Ryder, 2003; Viney *et al.*, 2005). It ignores the fact that an immune response may be parasite-species specific (Schmid-Hempel, 2003; Schmid-Hempel & Ebert, 2003; Adamo, 2004). There is likely to be some overlap in the immune components that a host uses to fight infection, given the diversity of parasites it may potentially encounter. However, in many cases it is not known, and therefore cannot be assumed *a priori*, which immune components play the most important role in determining resistance to a particular parasite, or even whether the

immune system is the major contributor to variation in resistance, relative to non-immune forms of host defence such as avoidance behaviour (Hart, 1994) or physical barriers (Wilson *et al.*, 2001). Moreover, it is often unclear how large a change is needed in an immune assay before one can infer a statistically significant change in parasite resistance (Keil *et al.*, 2001; Adamo, 2004). The few studies that have explicitly examined the relationship between a single component of the immune system and parasite resistance in natural host populations have generated mixed results. For instance, Schwarzenbach & Ward (2007) studied the association between phenoloxidase (PO), an important component of the insect immune system, and resistance to mites and entomopathic nematodes among replicate lines of yellow dung flies, *Scathophagia stercoraria* selected for different levels of PO, but found that PO level was not a good predictor of parasite resistance. Auld *et al.* (2010) found that high basal levels of haemocytes, an immune cell of invertebrates, served as a marker for susceptibility rather than resistance to the bacterial parasite *Pasteuria ramosa* in *Daphnia magna*. On the other hand, Kraaijeveld *et al.* (2001) recorded a positive correlation between haemocyte concentration and resistance to a parasitoid wasp, *Asobara abida*, in *Drosophila melanogaster*. Therefore, the relationship between parasite resistance and the immune response is likely to depend on the host-parasite interaction and the immune component that is being measured. Moreover, the sign and strength of the relationship may vary among host populations, or the pattern within populations may be different from the pattern across populations (Mucklow *et al.*, 2004). Hence, multiple host populations should be incorporated into such studies to gain a general impression of the relevance of the immune response to parasite resistance.

Here, using lab-reared three-spined sticklebacks, *Gasterosteus aculeatus*, from five populations from North Uist, Scotland and a widespread stickleback parasite, *Diplostomum spathaceum*, I carried out an artificial infection experiment to address whether there is (i) spatial variation in parasite resistance, (ii) spatial variation in immune response, and (iii) a relationship between parasite resistance and the immune response. Three-spined sticklebacks are an excellent species for investigating population divergence of ecologically-relevant traits (McPhail, 1994; McKinnon & Rundle, 2002). Since the last ice age, ancestral marine populations have repeatedly colonised freshwater environments. These invasion events have often been

accompanied by rapid evolutionary change and the divergence of morphological (Peichel *et al.*, 2001; Berner *et al.*, 2008), life history (Snyder & Dingle, 1989; Baker, 1994) and behavioural (Boughman, 2001; Bolnick *et al.*, 2009) traits. Differences in parasite faunas between freshwater and saltwater habitats may be an important source of selection driving divergence of these traits and mediating adaptation to freshwater (MacColl & Chapman, 2010). Moreover, parasite communities of sticklebacks have been shown to vary substantially among populations (Kalbe *et al.*, 2002; Barber, 2007, Chapter 3). Therefore, divergent parasite-mediated selection is likely to be a pervasive process that has important consequences for the evolution of immune responses and parasite resistance in stickleback populations.

Diplostomum spp. are digenean trematodes that use a range of fish species, including three-spined sticklebacks, as a second intermediate host in their three-host life cycle (Chappell, 1995). Fish become infected through exposure to cercariae, released from the first intermediate host (a snail), which penetrate the gills and skin and migrate to the eye lens or retinal tissue. They remain in the eye until infected fish are eaten by birds. Three lines of reasoning make the stickleback-*Diplostomum* interaction a good model for testing the three questions outlined above. Firstly, *Diplostomum* spp. are known to exert strong selective effects on their hosts. For instance, field data have shown that heavily infected individuals are selectively removed from the population (Pennycuik, 1971b; Lester, 1977; Kennedy, 1984). In the case of *D. spathaceum*, the parasite may cause direct host mortality (Brassard *et al.*, 1982). However, most parasite-induced host mortality is probably indirect and mediated through the parasite's detrimental effects on fish vision (Shariff *et al.*, 1980). Even at low levels of infection *Diplostomum* can affect the vision of sticklebacks (Owen *et al.*, 1993). Impaired vision in turn reduces foraging efficiency (Crowden & Broom, 1980) and anti-predator behaviour (Seppälä *et al.*, 2004). Therefore, fish from populations with a high prevalence and abundance of *Diplostomum* may be under strong selection to evolve resistance. Secondly, we have some understanding of the immune response to *Diplostomum* (Bortz *et al.*, 1984; Stables & Chappell, 1986b; Whyte *et al.*, 1987; Whyte *et al.*, 1989; Höglund & Thuvander, 1990; Whyte *et al.*, 1990). Of the immune mechanisms that have been identified as being important in fighting *Diplostomum* infection, the respiratory burst response probably constitutes the strongest candidate for killing the parasite prior to its establishment in the eye (Whyte *et al.*, 1989). The

respiratory burst is a cellular innate immune response mediated by granulocytes such as neutrophils, and is initiated upon contact with parasite antigen. Granulocytes increase their oxygen consumption, which stimulates the production of damaging reactive oxygen species (Secombes, 1996; Dahlgren & Karlsson, 1999; Alvarez-Pellitero, 2008). In this study, the proportion of granulocytes and the intensity of respiratory burst response served as measures of the innate immune response. Although the respiratory burst response is potentially vital in the response to *Diplostomum* infection, it has never been quantified to what extent variation in this immune parameter corresponds to variation in actual resistance to *Diplostomum*. Lastly, several studies have reported spatial variation in infectivity among *Diplostomum* strains (Ballabeni & Ward, 1993; Voutilainen *et al.*, 2009) and parasite resistance among host populations (Kalbe & Kurtz, 2006), indicating that population divergence in host and parasite traits may be a common feature of the *Diplostomum*-host interaction.

In addition to investigating spatial variation in resistance to *D. spathaceum*, the immune response and the association between the two traits, the current study had three further objectives. First, the design of the experiment allowed me to examine whether previous parasite exposure reduces the number of parasites that establish following a second exposure. Some studies have demonstrated a boosting effect of prior exposure on *Diplostomum* resistance (Höglund & Thuvander, 1990; Whyte *et al.*, 1990; Karvonen *et al.*, 2005), whereas others have not (Kalbe & Kurtz, 2006). Therefore, it is worthwhile assessing the generality of a potential adaptive immune response to *Diplostomum* infection. Second, I attempted to link population-level variation in resistance to *D. spathaceum* and immune response in the artificial infection experiment to natural levels of *Diplostomum* infection. The extent to which divergent parasite-mediated selection, as inferred from variation in natural infection levels, shapes investment in parasite resistance (Corby-Harris & Promislow, 2008; Hasu *et al.*, 2009) and immune response (Lindström *et al.*, 2004; Whiteman *et al.*, 2006; Bryan-Walker *et al.*, 2007) among populations remains largely unknown for vertebrate-macroparasite systems. Lastly, this study assessed whether there is a growth cost associated with *D. spathaceum* infection. There is some evidence of the detrimental growth effects of *Diplostomum*, but this is based solely on field data (Pennycuick, 1971b; Marcogliese *et al.*, 2001b) or correlative data from artificial

infections (Buchmann & Uldal, 1994). Only a handful of studies have examined growth costs formally by comparing the growth of exposed and unexposed hosts in an experimental setup (Ballabeni, 1994; Karvonen & Seppälä, 2008), and this has yet to be tested in the stickleback-*Diplostomum* interaction.

Even though *D. spathaceum* is not found in sticklebacks on North Uist (*D. gasterostei* is the native *Diplostomum* species), there is likely to be an overlap between the host immune response they induce, and therefore, in the potential mechanisms conferring resistance. The key feature that differentiates *D. spathaceum* and *D. gasterostei* is the infection site within the fish host: *D. gasterostei* and *D. spathaceum* infect the retinal tissue and lens, respectively (Karvonen *et al.*, 2006). Furthermore, use of *D. spathaceum* instead of *D. gasterostei* removed the possibility of close coevolution between parasite and host populations. In effect, the experiment tests for an evolutionarily naive interaction, and may thus reveal some general patterns about the evolution of spatial variation in parasite resistance and the immune response.

5.2 Methods

5.2.1 Study populations and parasites

Fish were collected from five geographically isolated lochs on North Uist, Outer Hebrides, Scotland during May 2008. Fish were caught using minnow traps (Gee traps, Dynamic Aqua, Vancouver). These lochs were chosen to represent a range of natural *Diplostomum gasterostei* infection levels (Table 5.1). For each population, F_1 offspring used in infection experiments were obtained by making 8 sets of unrelated full-sib crosses (families) from wild-caught fish. To make a cross, eggs were stripped from a gravid female and placed into a Petri dish containing a small volume of 1‰ salt solution. Males were killed, by overdose of anaesthetic (400 mg L⁻¹ MS222), and dissected to remove testes. Fine forceps were used to tease apart testes and release sperm, which was gently mixed with the eggs (Barber & Arnott, 2000). Two to three hours later, fertilisation was confirmed by low-power microscopy, and testes were removed from the fertilised clutches. Fertilised eggs were transferred to a falcon tube containing 50 mL of 1‰ salt solution. Eggs were then transported on ice to aquaria at

the University of Nottingham, where they were placed in a plastic cup with a mesh screen on the bottom suspended in a well-aerated tank containing dechlorinated water (Marchinko & Schluter, 2007). Water was treated with Methylene blue to reduce fungal infection. After 10 days, egg cups were transferred to individual half-tank partitions of 100 L tanks and the eggs were allowed to hatch. Following hatching, full-sib families were thinned to groups of 15. Clutches from each population were distributed haphazardly between tanks across the temperature-controlled room ($13.5^{\circ}\text{C} \pm 1^{\circ}\text{C}$). Fry were fed with infusoria (*Colpidium* spp.) for the first five days, then daily with brine shrimp (*Artemia salina*) naupliae until day 65 post-hatching. Thereafter, fish received chironomid larvae ('bloodworm'; defrosted from frozen) daily. Fish were maintained at a daylight regime mimicking the natural photoperiod on North Uist.

Pond snails (*Lymnaea stagnalis*) infected with *Diplostomum*, were collected from the lower pond of Jubilee Campus, University of Nottingham ($52^{\circ}57'\text{N}$; $1^{\circ}11'\text{W}$) in September 2008. This pond contains a large resident population of three-spined sticklebacks that carries high burdens of *Diplostomum* (personal observation). Dissections of the eyes from a small sample of sticklebacks revealed that *Diplostomum* was present only in the lens. No attempt was made to formally identify the species of *Diplostomum* metacercariae in sticklebacks (and the cercariae in snails) in Jubilee Campus. Two closely related and morphologically similar species, *D. spathaceum* Rudolphi, 1819 and *D. pseudospathaceum* Niewiadomska 1984, can infect the lens of sticklebacks. Although *L. stagnalis* may act as a first intermediate host for both species, in the U.K. only *D. spathaceum* cercariae have been recorded from *L. stagnalis* (Morley *et al.*, 2001); therefore the species in Jubilee campus sticklebacks was regarded to be *D. spathaceum*. A detailed taxonomic study should be carried out to verify the identification of this species. Snails were housed in plastic tanks containing dechlorinated water in a temperature controlled room ($16 \pm 1^{\circ}\text{C}$) under a 16L:8D photoperiod.

Population	<i>D. gasterostei</i> abundance	<i>D. gasterostei</i> prevalence	N	Geographic location
Chadha Ruaidh	9.82 ± 2.14	95.2 (76.7, 99.8)	21	57°35"N; 7°11"W
Hosta	1.59 ± 0.35	35.0 (16.7, 57.6)	20	57°37"N; 7°29"W
Lochmaddy	0	0 (0.0, 13.9)	24	57°36"N; 7°10"W
Reivil	2.73 ± 0.70	72.7 (40.5, 92.1)	11	57°36"N; 7°30"W
Tormasad	1.2 ± 0.38	45.0 (24.4, 68.0)	20	57°33"N; 7°19"W

Table 5.1 The five stickleback populations from North Uist used in the study. *D. gasterostei* prevalence and abundance data were obtained in May 2008 by counting the number of parasites in the left eye of approximately 20 sticklebacks from each loch. Abundance values are given with the standard error of the mean whereas prevalence values are given with 95% confidence intervals.

5.2.2 Experimental design

The infection experiment consisted of two rounds of exposure and one round of dissection (Fig. 5.1). Fish were six months old at the time of the first round of exposure (November 2008). The second round of exposure took place in January 2009, six weeks after the first round. This gap was chosen to give previously exposed fish sufficient time to develop an adaptive immune response, which can take up to several weeks in teleost fish (Jones, 2001; Magnadóttir, 2006). If there is a boosting effect of a previous exposure on subsequent parasite resistance, I predicted to see a reduction in the number of established parasites after the second round of exposure. All fish were dissected two days after the second round of exposure to determine the number of *D. spathaceum* metacercariae in the lens and to obtain morphological and immunological measurements. Fish have a very narrow window of time, typically 24 hours, to mount an immune response to *Diplostomum* and eliminate the parasite prior to its migration to the eye lens (Chappell, 1995). Therefore, by minimising the amount of time between the second round of exposure and immunological measurements, it increases the chance of detecting a still-active and measurable innate immune response (Kalbe & Kurtz, 2006).

The experiment utilised a split-plot design to compare three parasite exposure treatments (control unexposed (C), once-exposed (1x) and twice-exposed (2x)) among five stickleback populations. The total sample size of the experiment was 120 fish. For each population, fish from 8 full-sib families were used, such that one fish per family was assigned to each of the three treatments; however, for Hosta only 7 full-sib families were available. As the number of fish that could be processed for immunological measurements was limited to a maximum of 32 per day by logistical constraints, the experiment was split into four blocks of 30 fish. Populations and treatments were balanced across blocks. However, families could not be balanced across blocks and were therefore randomly assigned to a block. To enable the comparison of parasite treatments within families, each block comprised two families.

Throughout the experiment, fish were housed in groups of 15 in half-tank partitions of 100 litre tanks. One family per population was present in each partition such that a tank housed all fish belonging to the same block. Families were assigned randomly to blocks and fish within families were assigned randomly to treatments. Two weeks prior to the first round of exposure, fish were injected subcutaneously with a fluorescent visible implant elastomer tag (Northwest Marine Technology, Shaw Island, WA) to allow identification of individuals. Within partitions, each fish was given a unique mark. Fish were fed bloodworm (defrosted from frozen) to satiation daily and were maintained in a temperature controlled room ($13.5^{\circ}\text{C} \pm 1^{\circ}\text{C}$) on a 8L:16D cycle.

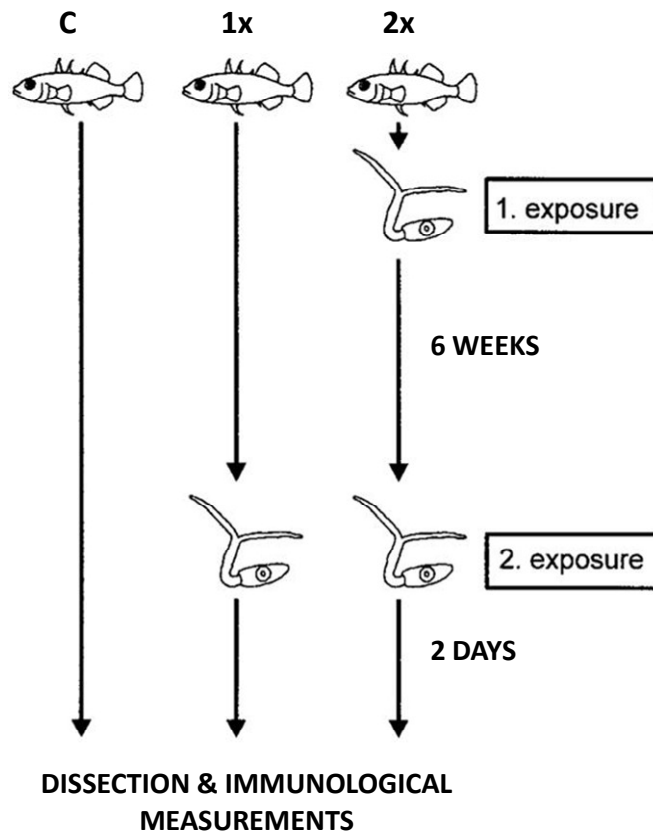


Figure 5.1 The design of the *D. spathaceum* infection experiment. The experiment comprised two rounds of exposure and one round of dissection. The figure is modified from Kalbe & Kurtz (2006).

5.2.3 Infection protocol

D. spathaceum cercariae were obtained by placing snails in individual 50 mL falcon tubes containing 40 mL dechlorinated tap water and exposing them to a cold fluorescent light for one hour to stimulate shedding. Three 1 mL samples were taken and the number of cercariae counted using a dissection microscope. Total cercarial densities were then calculated from the average of the three samples. Cercariae from four snails were mixed in equal proportion to obtain a cercarial suspension containing several *D. spathaceum* genotypes (infected snails typically contain 2-4 *Diplostomum* genotypes (A. Karvonen, personal communication)). From the mixed-genotype suspension, groups of 20 cercariae were isolated in small Petri dishes. The same four snails were used throughout the experiment to minimise potentially confounding

effects of parasite genotype on resistance and measures of host immune response. However, as one of the snails died before the second round of infections, fish from this group were exposed to cercariae from three snails only.

One hour prior to exposure, fish were measured (to the nearest 0.1mm) and weighed (to the nearest mg), transferred to individual three litre plastic tanks containing one litre of dechlorinated water and allowed to acclimatise. A Petri dish containing 20 cercariae was then added to each tank and gently agitated to ensure that cercariae were released into the water. Control fish were sham-exposed by adding a Petri dish containing a small volume of dechlorinated water. Seven hours post-exposure, fish were removed from their individual tanks and returned to their original group tank. Fish in the twice-exposed group were re-exposed in an identical manner six weeks later. Throughout the experiment, if fish were found in a state of poor health, as defined by reduced feeding activity and cessation of movement, they were euthanised by overdose of MS222 (400 mg L⁻¹) and destruction of the brain. By the end of the experiment, a total of 7 fish had been euthanised. However, in only one case was this associated with parasite exposure.

5.2.4 Dissection and immunological assays

Two days after the second round of exposure all fish were killed, by overdose of MS222 (400 mg L⁻¹) and destruction of the brain, and were measured and weighed as before. Both eyes were dissected out and the number of metacercariae in the lenses determined using a dissection microscope. In twice-exposed fish, metacercariae from the first and second round of exposure could be easily differentiated on the basis of worm size. Infection levels from the first round of exposure were determined only at the end of experiment. However, as the eye lens constitutes an immunologically privileged site, worm mortality in the six-week interval is unlikely to have affected worm counts (Chappell, 1995).

As measures of cellular innate immune system, the proportion of granulocytes and the respiratory burst response were quantified. Extraction of head kidney leukocytes (HKL) and quantification of HKL cell numbers followed a protocol modified after

Scharsack et al. (2004). Head kidneys were dissected out using fine forceps. HKLs were isolated by squeezing head kidneys through a 40 µm cell strainer (BD-Falcon) into a 50 mL falcon tube containing 2 mL 'R-90'. R-90 is RPMI-1640 (without phenol red, Gibco) diluted with 10% v/v distilled water, a medium that matches stickleback osmolarity (Scharsack *et al.*, 2004). Cells were washed once by centrifugation at 4°C and resuspended in a final volume of 500 µL R-90. Cells and media were kept on ice throughout the protocol.

HKL numbers were quantified by a standard cell dilution assay. To individual flow cytometry tubes the following were added: 50 µL of washed HKLs, 100 µL R-90, 5×10^4 green fluorescent beads (3 µm, Polyscience) and propidium iodide (2 mg L⁻¹, Sigma-Aldrich). Propidium iodide is a DNA-binding substance that enables the detection of dead cells. Using a flow cytometer (FACS, Becton & Dickinson), FSC/SSC characteristics of at least 10,000 events were acquired at linear scale; fluorescent intensities at wavelengths of 530 (beads) and 585nm (propidium iodide) were acquired at log scale. Data were analysed using FACS Diva software. Cellular debris was easily distinguished from viable cells by its low FSC values and was excluded from subsequent analyses. Likewise, fluorescent beads (green fluorescence positive) were easily differentiated from viable HKLs (propidium iodide negative, green fluorescence negative) and dead HKLs (propidium iodide positive, green fluorescence negative). The total number of viable HKLs per sample was then calculated as: $N[\text{HKL}] = \text{events}[\text{viable HKL}] \times \text{number}[\text{fluorescent beads}] / \text{events}[\text{fluorescent beads}]$. To standardise cell volumes for the respiratory burst assay, HKL suspensions were adjusted to a final concentration of 6.25×10^5 cells mL⁻¹. The flow cytometric data were also used to calculate ratios of granulocytes to lymphocytes, which have characteristically high SSC and low SSC profiles, respectively (Scharsack *et al.*, 2004; Serada *et al.*, 2005).

Respiratory burst activity was measured using an *in vitro* luminol-enhanced chemiluminescence assay. The luminol buffer was prepared by adding luminol dissolved in 0.1M NaOH (10 mg mL⁻¹) to a buffer solution (25 µL of dissolved luminol per ml of Hanks' buffered saline solution (HBSS, Gibco), containing 20mM HEPES (Sigma-Aldrich) and 1 g mL⁻¹ bovine serum albumen (BSA, Sigma-Aldrich)). 20 µL of luminol buffer was added to 160 µL of HKL suspension (10^5 cells per well) in wells of

96-well microtitre plate. To allow the cells to take up luminol, plates were incubated at room temperature for 30 minutes. Luminol is a molecule that reacts with secreted and intracellular reactive oxygen species (ROS), and emits light when excited molecules return to their ground state (Stenfeldt & Dahlgren, 2007). In the current assay, production of reactive oxygen species (ROS) was stimulated by the addition of 20 μL of a zymosan suspension (7.5 g L^{-1} phosphate-buffered saline (PBS, Gibco)). When enough cells were available, a duplicate zymosan treatment and a control PBS-only treatment were also included. The respiratory burst reactions were measured at room temperature at three minute intervals for two hours using a microtitre plate luminometer (Berthold Technologies). Relative luminescence (RLU) was tracked using WinGlow software (Berthold Technologies). The amount of light emitted in a given reaction therefore provides a quantitative measure of the number of ROS produced by HKLs in a sample. From each reaction curve, the peak of the respiratory burst response was determined (hereafter, 'respiratory burst'). On two of the four days (blocks) of immunological analysis, the luminometer had to be re-started mid-reaction due to a technical difficulty. As a result, the readings immediately before, during and after the peak were not recorded for several fish from these blocks and the true peak values could not be obtained. However, the value of the peak for these reactions could be determined by approximation. The reliability of this approximation technique was confirmed by deleting data between the identical time interval from reactions for fish from both blocks that were not affected by the technical problem and comparing approximations and true peak values (correlation between both values: $r = 0.99$).

5.2.5 Statistical analysis

5.2.5.1 Natural *D. gasterostei* infection levels

All statistical analyses were conducted in GenStat (release 12, VSN International Ltd., Hemel Hempstead, U.K.). A generalised linear model (GLM) with a negative binomial error distribution and a logarithm link function was used to analyse variation in natural abundance of *D. gasterostei*. Population was the only fixed effect in this model.

5.2.5.2 Resistance to *D. spathaceum*

D. spathaceum burdens resulting from exposure in the artificial infection experiment ('number of metacercariae' as the response variable) were modelled using generalised linear mixed models (GLMMs) with a binomial error structure and a logit link function. The number of binomial totals was 20, the exposure dose. The number of metacercariae was interpreted to be inversely proportional to resistance to *D. spathaceum*. Four different models were used to explore different subsets of the data. (i) To examine variation in the number of metacercariae after the first round of exposure, a fixed model with the effects sex, length before the first round of infection ('initial length') and population was used. (ii) To examine variation in the number of metacercariae after the second round of exposure, the fixed model consisted of the effects sex, length at the end of the experiment ('final length'), population, treatment (1x /2x) and the interaction between treatment and population (treatment \times population). (iii) To examine variation in the number of metacercariae after a single exposure to 20 *D. spathaceum* cercariae, the following fixed model was used: treatment (1x/2x), population and treatment \times population. This model tested for a difference in the number of metacercariae using data from the first exposures of once-exposed fish (in the second round of exposure) and twice-exposed fish (in the first round of exposure). (iv) Data from twice-exposed fish were used to test for an effect of previous exposure on the number of metacercariae in the second exposure. The fixed model included the effects exposure round, population and exposure round \times population.

In all models block, and family nested within population (population \times family) were included as random effects. This latter effect controls for variation among families. The significance of random effects was assessed via likelihood ratio tests (Galwey, 2006), which compare the differences in the deviance of the reduced model, without the random effect, and the full model to a χ^2 distribution with one degree of freedom. Minimum adequate models (MAM) were constructed by stepwise deletion, i.e. the sequential dropping of non-significant fixed effects (Crawley, 2007). In GLMMs, significance of fixed effects was assessed by Wald F tests. In GLMs, significance of fixed effects was assessed by noting the change in deviance, compared to a χ^2

distribution with the appropriate degrees of freedom. If main effects were marginal to interaction effects, significance of main effects was tested in the presence of interactions.

5.2.5.3 Immune measures

Immunological data were analysed using linear mixed models (LMMs). A first model analysed variation in the proportion of granulocytes ('prop gran' as the response variable, log-transformed to achieve normality). The fixed model consisted of the effects sex, final length, treatment, population and treatment \times population. A second model was used to analyse variation in the respiratory burst response ('respiratory burst' as the response variable, log-transformed to achieve normality). Sex, final length, treatment, population, prop gran, and treatment \times population were included as fixed effects. One fish was excluded from the respiratory burst model owing to an insufficient number of HKLs. In both models, block, and family nested within population (population \times family) were fitted as random effects. If neither effect was significant, the model reverted to a general linear model. As above, significance of fixed effects was assessed via stepwise regression by sequential deletion of non-significant effects until a MAM was specified.

5.2.5.4 Growth and *D. spathaceum* infection

A GLMM with normal errors was used to examine variation in specific growth rate (SGR; *sensu* Barber, 2005) of individual fish. SGR, the average daily percentage increase in length, was calculated using the equation: $SGR = 100 * [\ln(L_{t1}/L_{t0})]/(t_1 - t_0)$, where L_{t1} and L_{t0} represent the standard length measure before, and at the end of, the experimental infection, and $t_1 - t_0$ denotes the number of days between measurements. This was 45, 46, 47 and 48 for block 1, 2, 3 and 4, respectively. Block and family nested within population were fitted as random effects. The fixed model included the effects sex, initial length, infection, population and infection \times population. The effect 'infection' comprised two levels: 'i', which included infected 2x-fish, and 'o', which included uninfected 2x fish, 1x fish and control fish. Therefore, this effect tests whether there is a somatic growth cost associated with *D. spathaceum* infection. 1x-

fish were also included in the 'o' level because the impact of infection resulting from the second round of exposure, two days prior to dissection, was probably negligible. To investigate the actual impact of *D. spathaceum* infection on growth, the relationship between the SGR and number of metacercariae of infected 2x-fish was analysed with Pearson's correlations, separately for each population.

5.2.5.5 Associations between natural *D. gasterostei* infection, resistance to *D. spathaceum* and the innate immune response

Pearson correlation analysis was used to examine the relationship at the population level between *D. gasterostei* infection in the wild (natural abundance rank score), and *D. spathaceum* resistance and log-transformed respiratory burst response. An increase in the abundance rank score corresponds to an increase in mean natural *D. gasterostei* abundance (Table 5.1). Resistance to *D. spathaceum* was defined as the mean number of metacercariae resulting from one exposure to 20 *D. spathaceum* cercariae (data from the 1x and 2x treatments were pooled); resistance is inversely proportional to the number of metacercariae. Since there was no significant effect of experimental treatment on the respiratory burst response, data from C, 1x and 2x treatments were pooled. To investigate the relationship between *D. spathaceum* resistance and the respiratory burst response, family-level means of both measurements were correlated. A two-tailed test was used to assess the significance of correlation coefficients.

5.3 Results

5.3.1 Natural *D. gasterostei* infection

There was significant variation in *D. gasterostei* abundance among populations ($\chi^2_4 = 26.46$, $P < 0.001$; Table 5.1). *D. gasterostei* was absent from Lochmaddy.

5.3.2 Resistance to *D. spathaceum*

For both rounds of exposure, there was no significant effect of block on the number of metacercariae (first round LR test: $\chi^2_1 = 0.00$, $P >> 0.05$, variance component estimate \pm

S.E. = 0.008 ± 0.090 ; second round LR test: $\chi^2_1 = 0.00$, $P \gg 0.05$, variance component estimate \pm S.E. = 0 ± 0), therefore data from all blocks were pooled. The number of metacercariae varied significantly among fish families after the first and second round of exposure (first round LR test: $\chi^2_1 = 0.00$, $P \gg 0.05$, variance component estimate \pm S.E. = 0.29 ± 0.22 ; second round LR test: $\chi^2_1 = 2.89$, $P = 0.045$, variance component \pm S.E. = 0.12 ± 0.11). Population predicted the number of metacercariae in the first ($F_{4,30.0} = 23.00$, $P < 0.001$; Fig. 5.2a) and second round of exposure ($F_{4,66.0} = 56.53$, $P < 0.001$; Fig. 5.2b). Lochmaddy consistently had the highest number of metacercariae relative to the other four populations. After the first round of exposure, Reivil had the lowest number of metacercariae relative to Chadha Ruaidh, Hosta or Tormasad. This small scale variation among Reivil, Chadha Ruaidh, Hosta and Tormasad was less pronounced in once-exposed (1x) fish in the second round of infection; the rank of infection success changed such that Hosta had the lowest mean number of metacercariae (Fig. 5.2b). There was no significant effect of initial length ($F_{1,29.0} = 1.09$, $P = 0.305$) or sex ($F_{1,29.0} = 0.28$, $P = 0.597$) on the number of metacercariae after the first round of exposure. After the second round of exposure, the number of metacercariae did not differ significantly between once-exposed (1x) and twice-exposed (2x) fish ($F_{1,65.0} = 1.98$, $P = 0.165$), and this was consistent across populations (treatment \times population: $F_{4,32.0} = 8.14$, $P = 0.113$). Likewise, final length ($F_{1,58.1} = 0.71$, $P = 0.402$) and sex ($F_{1,57.2} = 0.71$, $P = 0.391$) did not significantly influence the number of metacercariae resulting from the second round of exposure.

Comparing data from 1x fish from the second round of exposure and 2x fish from the first round of exposure (i.e. first exposures), the number of metacercariae varied significantly among fish families (LR test: $\chi^2_1 = 6.38$, $P = 0.006$, variance component estimate \pm S.E. = 0.15 ± 0.11) but not among blocks (LR test: $\chi^2_1 = 0.00$, $P \gg 0.05$, variance component estimate \pm S.E. = 0 ± 0). Population predicted the number of metacercariae ($F_{4,33.7} = 48.89$, $P < 0.001$; Fig. 5.3a). The number of metacercariae differed significantly between treatments ($F_{1,36.1} = 6.57$, $P = 0.015$), with 2x fish after the first round of exposure having a higher number of metacercariae than 1x fish after the second round of exposure (mean \pm S.E.: 1x = 3.59 ± 0.73 , 2x = 4.43 ± 0.87). This effect was similar across populations (treatment \times population: $F_{4,30.4} = 2.15$, $P = 0.098$). Comparing data from twice-exposed fish only, there was a significant effect of fish

family (LR test: $\chi^2_1 = 12.77$, $P < 0.001$, variance component estimate \pm S.E. = 0.22 ± 0.13) but not of block (LR test: $\chi^2_1 = 0.00$, $P >> 0.05$, variance component estimate \pm S.E. = 0 ± 0) on the number of metacercariae. The number of metacercariae varied significantly among populations ($F_{4,31.9} = 36.77$, $P < 0.001$; Fig. 5.3b) and differed significantly between rounds of exposure ($F_{1,37.8} = 13.14$, $P < 0.001$; Fig. 5.3b), being lower in the second round of exposure (mean \pm S.E: first round = 4.43 ± 0.87 , second round = 2.71 ± 0.64). This effect of exposure round was consistent across populations (exposure round \times population: $F_{4,34.2} = 0.12$, $P = 0.976$).

5.3.3 Immune measures

Block and fish family did not have a significant effect on the proportion of granulocytes (block LR test: $\chi^2_1 = 1.08$, $P = 0.149$, variance component estimate \pm S.E. = 0.0013 ± 0.0019 ; population \times family LR test: $\chi^2_1 = 2.39$, $P = 0.061$, variance component estimate \pm S.E. = 0.0036 ± 0.0027). The proportion of granulocytes did not differ significantly among treatments ($F_{2,101} = 0.78$, $P = 0.462$) and populations ($F_{4,103} = 1.57$, $P = 0.189$), nor was there a significant interaction between treatment and population ($F_{8,93} = 0.89$, $P = 0.528$, Fig. 5.4a). The proportion of granulocytes did not differ significantly between males and females ($F_{1,108} = 0.83$, $P = 0.366$) and was not associated with final length of fish ($F_{1,107} = 0.32$, $P = 0.574$). The respiratory burst response varied significantly among fish families (LR test: $\chi^2_1 = 4.43$, $P = 0.018$, variance component estimate \pm S.E. = 0.011 ± 0.010) and among blocks (LR test: $\chi^2_1 = 9.43$, $P = 0.001$, variance component estimate \pm S.E. = 0.0077 ± 0.0045). Population explained significant variation in respiratory burst ($F_{4,34.3} = 2.87$, $P = 0.038$; Fig. 5.4b). The respiratory burst was highest in Chadha Ruaidh; Chadha Ruaidh was also the only population in which exposed fish exhibited higher respiratory burst values than control fish (Fig. 5.4b). Nevertheless, neither treatment ($F_{2,69.5} = 0.73$, $P = 0.486$) nor treatment \times population ($F_{8,61.1} = 1.55$, $P = 0.158$) explained significant variation in respiratory burst. Respiratory burst was significantly influenced by the proportion of granulocytes ($F_{1,98.9} = 15.20$, $P < 0.001$) and final length of fish ($F_{1,100.1} = 7.77$, $P = 0.006$); both covariates were positively correlated with respiratory burst (parameter estimate \pm SE: prop gran = 0.620 ± 0.210 , length = 0.019 ± 0.008). Respiratory burst did not differ significantly between males and females ($F_{1,99.3} = 2.60$, $P = 0.110$).

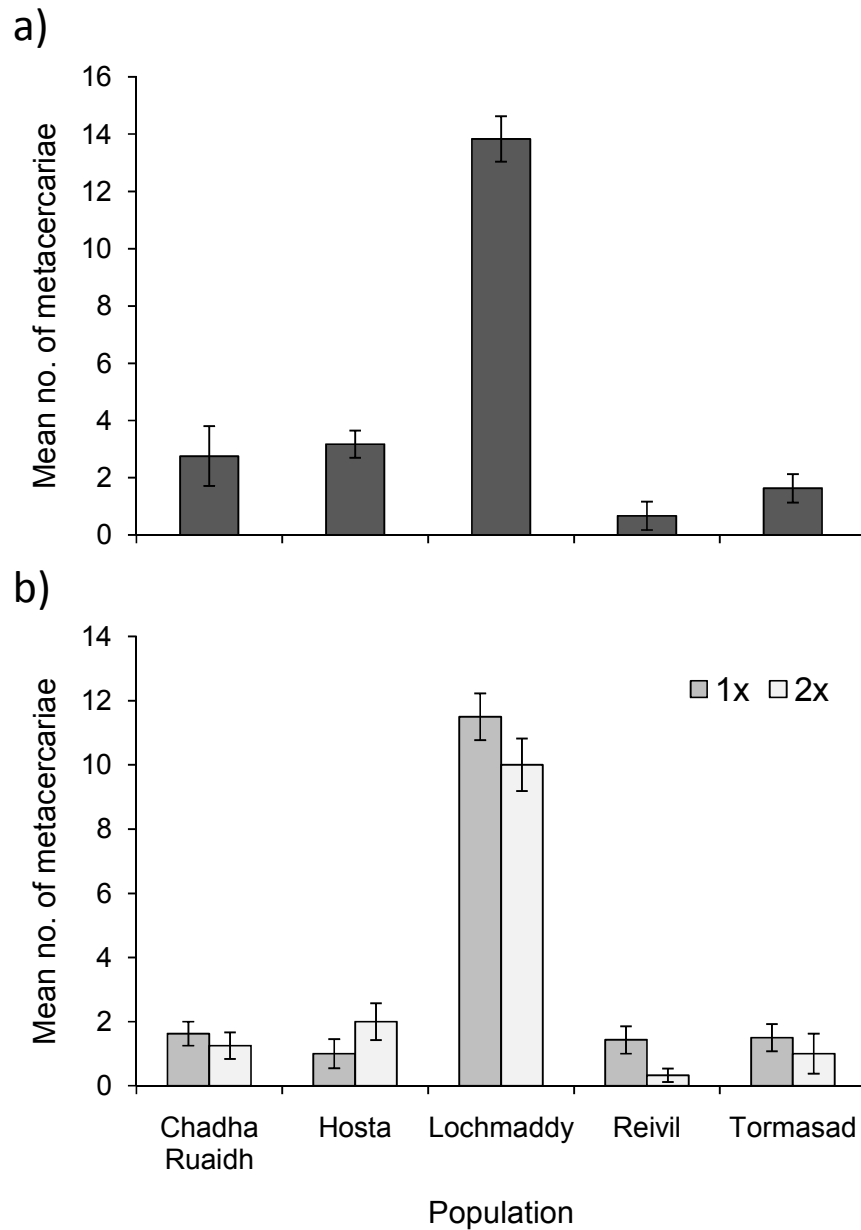


Figure 5.2 Variation in *D. spathaceum* burdens among the five stickleback populations in the infection experiment. Bars represent the mean number of metacercariae (with standard errors) following exposure of individual fish to 20 *D. spathaceum* cercariae. a) First round of exposure (twice-exposed fish only) and b) second round of exposure (once-exposed (1x) and twice-exposed (2x) fish). In a) metacercariae are six weeks old, whereas in b) metacercariae are two days old. The number of metacercariae is interpreted to be inversely proportional to *D. spathaceum* resistance.

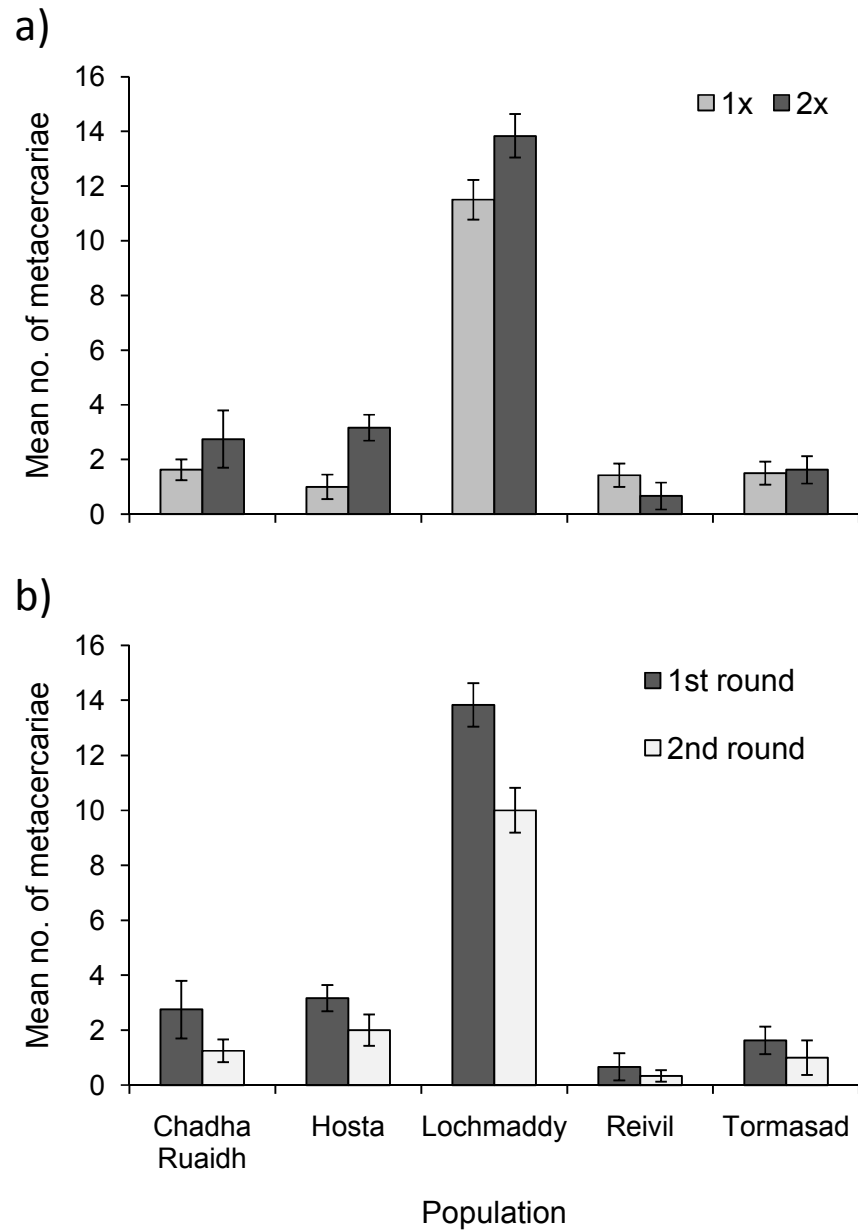


Figure 5.3 Further population-level comparisons of variation in experimental *D. spathaceum* burdens between: a) first exposures of once-exposed (1x) and twice-exposed (2x) fish, and b) both rounds of exposure of twice-exposed fish. Bars represent the mean number of metacercariae (with standard errors) following exposure of individual fish to 20 *D. spathaceum* cercariae. In a) metacercariae are two days old (1x) and approximately six weeks old (2x), and in b) metacercariae are approximately six weeks old (1st round) and two days old (2nd round).

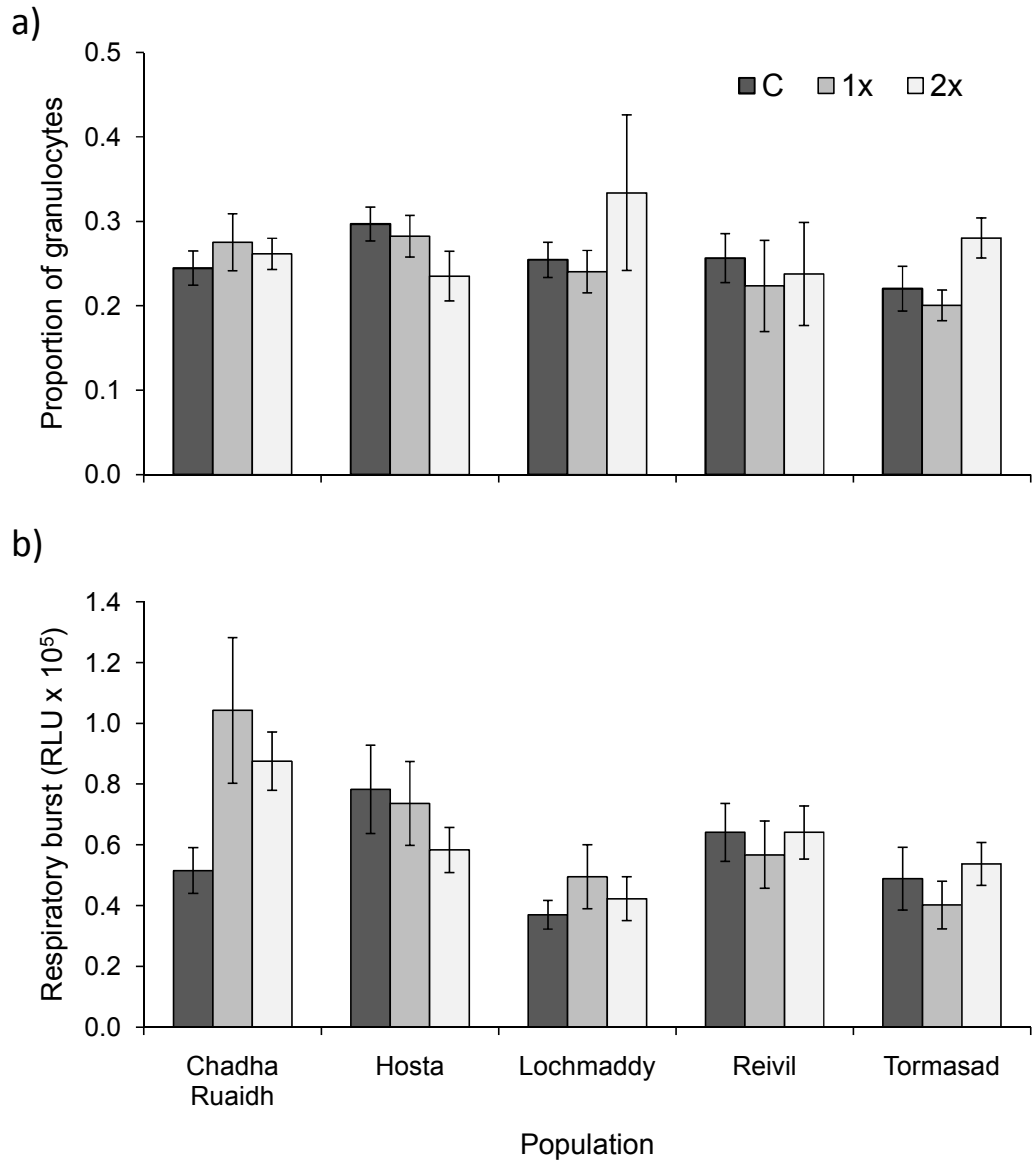


Figure 5.4 Variation in innate immune measures among the five stickleback populations in the *D. spathaceum* infection experiment: a) proportion of granulocytes and b) respiratory burst response. Measures were taken two days after the second round of exposure. The proportion of granulocytes was determined by flow cytometry, whereas measures of the respiratory burst response were obtained from a luminol-enhanced chemiluminescence assay. C, 1x and 2x denote the control, once-exposed and twice-exposed treatments respectively. Error bars are the standard error of the mean.

5.3.4 Growth and *D. spathaceum* infection

Specific growth rate (SGR) varied significantly among fish families (LR test: $\chi^2_1 = 10.81$, $P < 0.001$, variance component estimate \pm S.E. = 0.0015 ± 0.0007) but not among blocks (LR test: $\chi^2_1 = 0.05$, $P = 0.417$, variance component estimate \pm S.E. = 0.00005 ± 0.00025). SGR did not differ significantly between *D. spathaceum*-infected and uninfected fish ($F_{1,76.6} = 0.67$, $P = 0.415$) and this was consistent across populations (infection \times population: $F_{4,73.5} = 0.76$, $P = 0.556$; Fig. 5.5). However, SGR was significantly influenced by population ($F_{4,36.3} = 15.68$, $P < 0.001$; Fig. 5.5), initial length ($F_{1,98.2} = 15.68$, $P = 0.003$) and sex ($F_{1,91.1} = 25.54$, $P < 0.001$). Females grew faster than males (parameter estimate \pm S.E.: females = 0.326 ± 0.009 , males = 0.267 ± 0.011) and initial length was negatively correlated with SGR (parameter estimate \pm S.E. = -0.010 ± 0.003), such that larger fish grew proportionately slower. The sign of the relationship between SGR and the number of metacercariae varied among populations, but all correlations were non-significant (Chadha Ruaidh: $r = -0.52$, $P = 0.187$; Hosta: $r = 0.28$, $P = 0.592$; Lochmaddy: $r = -0.02$, $P = 0.966$; Reivil: $r = 0.13$, $P = 0.287$; Tormasad: $r = 0.06$, $P = 0.879$).

5.3.5 Associations between natural *D. gasterostei* infection, resistance to *D. spathaceum* and the innate immune response

At the population level, there was a negative but non-significant correlation between the mean number of *D. spathaceum* metacercariae in the infection experiment and the natural abundance rank score of *D. gasterostei* and ($r = -0.69$, $P = 0.197$; Fig. 5.6a). This effect was largely due to the Lochmaddy population. In contrast, log-transformed respiratory burst was almost significantly positively correlated with natural abundance rank score of *D. gasterostei* ($r = 0.84$, $P = 0.07$; Fig. 5.6b). At the family-level across populations, the correlation between log-transformed respiratory burst and the mean number of *D. spathaceum* metacercariae was negative and not significant ($r = -0.25$, $p = 0.137$, $n = 38$; Fig. 5.7).

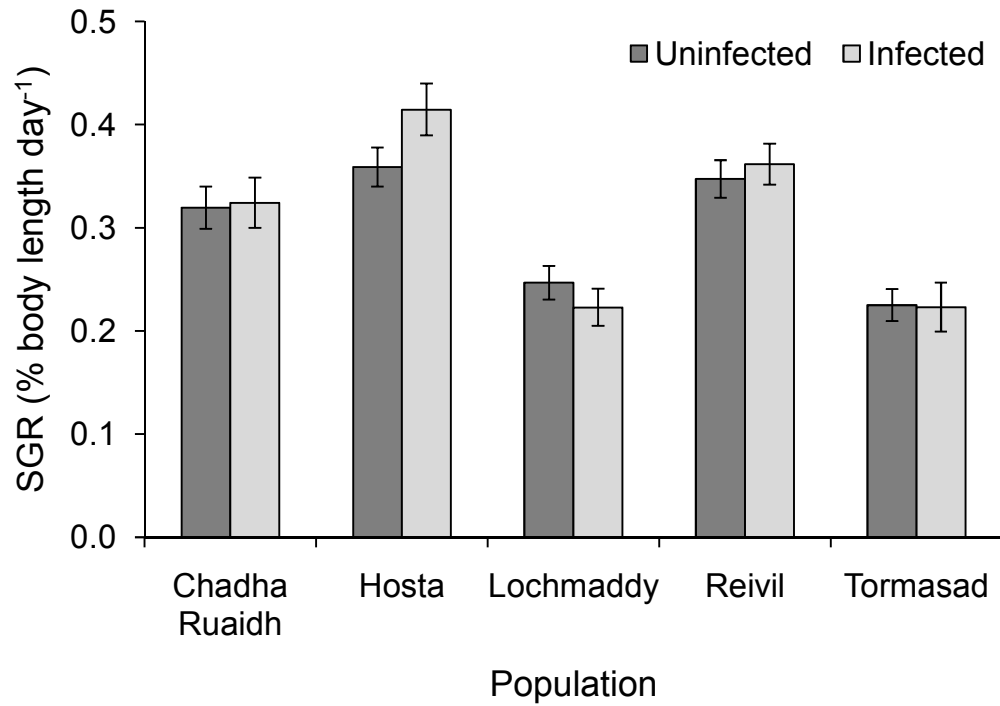


Figure 5.5 Differences in specific growth rate (SGR) between *D. spathaceum*-infected fish and uninfected fish across populations. SGR, a measure of the average daily percentage increase in fish length, was calculated using the equation: $SGR = 100 * [\ln(L_{t1}/L_{t0})] / (t_1 - t_0)$, where L_{t1} and L_{t0} represent the standard length measure before, and at the end of, the experimental infection and $t_1 - t_0$ denotes the number of days between measurements. Error bars represent the standard error of the mean.

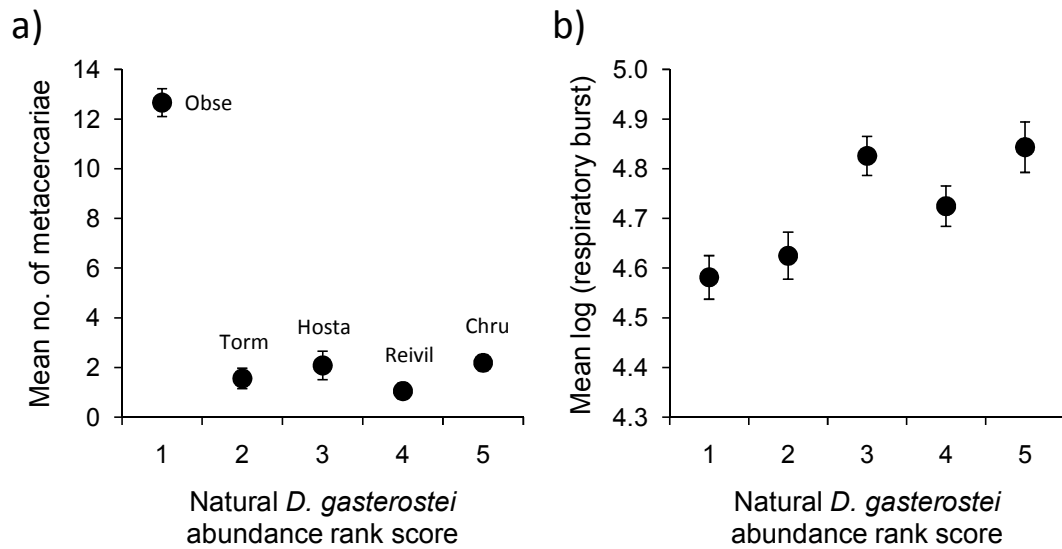


Figure 5.6 Relationship between population means of natural abundance of *D. gasterostei* and: a) number of *D. spathaceum* metacercariae in the infection experiment ($r = -0.69$, $P = 0.197$), b) log-transformed respiratory burst response ($r = 0.84$, $P = 0.073$). Natural abundance of *D. gasterostei* was estimated by sampling approximately 20 fish per loch in May 2008. Abundance rank score increase corresponds to an increase in natural *D. gasterostei* abundance. The number of metacercariae was calculated from pooled data of first exposures of 1x and 2x fish, and was interpreted to be inversely proportional to *D. spathaceum* resistance. Respiratory burst values are also means of data pooled from C, 1x and 2x fish. Obse = Lochmaddy, Torm = Tormasadh, Chru = Chadha Ruaidh. Error bars are the standard error of the population mean.

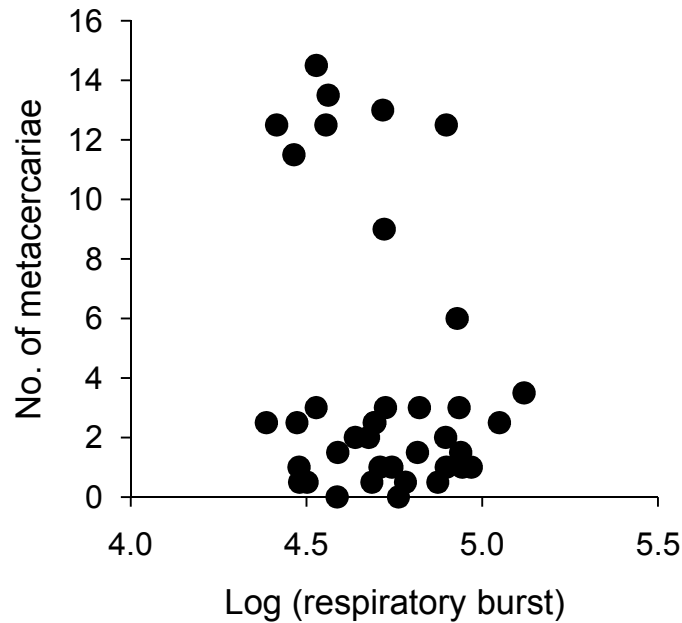


Figure 5.7 Relationship between family means of the number of *D. spathaceum* metacercariae in the infection experiment and log-transformed respiratory burst response ($r = -0.25$, $P = 0.137$).

5.4 Discussion

In spite of significant variation in both resistance to *Diplostomum spathaceum* and the respiratory burst response within and among stickleback populations, the respiratory burst response was not a good predictor of *D. spathaceum* resistance. Resistance to *D. spathaceum* was interpreted to be inversely proportional to the number of metacercariae following a single exposure to 20 cercariae. I selected the respiratory burst response as the immune parameter to measure because immunological studies have verified the ability of reactive oxygen species to kill the migrating stage *Diplostomum* (e.g. Whyte *et al.*, 1989) and because it can be quantified readily easily in sticklebacks (Kurtz *et al.*, 2004; Scharsack *et al.*, 2007b). Furthermore, since *Diplostomum* cercariae migrate to the eyes within 24 hours after penetrating fish skin (Whyte *et al.*, 1991; Chappell, 1995; Karvonen *et al.*, 2003), the host has a very short window of time in which to respond to the infection, and presumably an effective innate response is the only means of resistance following a single exposure. For these reasons, I expected the respiratory burst response to partially determine

resistance to *D. spathaceum*. However, no relationship consistent with such a mechanism was observed.

This is an important finding in the context of ecological immunology. A common assumption of ecological immunologists is that measures of the immune system accurately reflect, and are quantitatively linked to, an individual's ability to resist parasites (Sheldon & Verhulst, 1996; Owens & Wilson, 1999; Adamo, 2004; Lee, 2006). However, the number of studies that have explicitly tested this assumption in individuals from natural host populations under standardised conditions (Siva-Jothy, 1995) is limited (but see e.g. Mucklow *et al.*, 2004; Saks *et al.*, 2006; Schwarzenbach & Ward, 2007). To my knowledge, this experiment is the first to do so for a vertebrate-macroparasite interaction across multiple host populations. These results highlight the need for measuring multiple immune indices to obtain a more detailed understanding of the relationship between parasite resistance and the immune response, as others have argued (Keil *et al.*, 2001; Blount *et al.*, 2003; Adamo, 2004; Bradley & Jackson, 2008). The marked differences in *D. spathaceum* resistance among populations after one round of parasite exposure could only have been generated by genetically based differences in innate defence mechanisms (Kalbe & Kurtz, 2006; Rauch *et al.*, 2006a). Therefore, there may be other non-specific innate immune mechanisms mediating resistance to *Diplostomum* that have yet to be identified, such as complement, lysozyme, or natural antibodies (Whyte *et al.*, 1990; Jones, 2001; Magnadóttir, 2006; Alvarez-Pellitero, 2008). Alternatively, the protective mechanism may depend on non-immunological barriers such as fish skin thickness (Betterton, 1974), which may limit penetration of *Diplostomum* cercariae, or behavioural resistance (Karvonen *et al.*, 2004b). Moreover, any trade-off between investment in these other defence mechanisms and the respiratory burst response may further obscure the relationship between resistance to *D. spathaceum* and the immune system.

The significant difference among populations in *D. spathaceum* resistance was due mostly to one population, Lochmaddy, which was substantially more susceptible than the other four populations. Interestingly, this was the only population not naturally exposed to *D. gasterostei*. Because Lochmaddy is a saltwater loch, and the first intermediate host of *D. gasterostei* is a freshwater snail species (*Radix* spp.) that

cannot survive in marine conditions, the evolutionary naivety of this population is certain. High susceptibility to *Diplostomum* may be a general property of saltwater stickleback populations. Recently, MacColl and Chapman (2010) found that another saltwater population from North Uist, as well as a Canadian marine population, showed high susceptibility to *Diplostomum* in field transplant experiments. These data suggest that selection for resistance may be relaxed in the absence of the parasite (Webster *et al.*, 2004; Bryan-Walker *et al.*, 2007; Hasu *et al.*, 2009), especially if there are costs associated with the maintenance of parasite resistance (Rigby *et al.*, 2002; Zuk & Stoehr, 2002). In a comparable study of population-level variation in resistance to *Diplostomum pseudospathaceum* among two natural German stickleback populations, Kalbe & Kurtz (2006) documented a similar pattern: the unexposed population was markedly more susceptible than the population that was naturally exposed.

There was also small-scale variation in resistance to *D. spathaceum* among the four freshwater populations after the first round of exposure. This pattern was not repeatable in immunologically naive fish that were exposed in the second round of exposure (i.e. the once-exposed treatment). A possible explanation for this inconsistency is a change in the viability of cercariae isolated from the same snails between rounds of exposure (Kalbe & Kurtz, 2006). However, comparing resistance data from both rounds of exposure of twice-exposed fish suggested otherwise: the rank order of *D. spathaceum* resistance in the five populations was identical after both rounds of exposure. Therefore, the difference in the small-scale pattern of variation in parasite resistance between once-exposed fish and twice-exposed probably reflects intra-family and intra-population variation in resistance, rather than a true biological difference between the exposure rounds. Because this within-population variation has a genetic basis, it provides evidence for the *potential* of parasite-mediated selection (Little, 2002). Contrary to expectations, variation in *D. spathaceum* resistance among the four freshwater populations that are naturally infected was not correlated with variation in natural abundance of *Diplostomum gasterostei*. Assuming that differences in natural infection reflect different strengths of parasite-mediated selection, this suggests that divergent parasite-mediated selection cannot explain the small-scale variation in *D. spathaceum* resistance. Instead, differences among these four populations may be the result of general population-level variation in life history

architecture or trade-offs, generated by adaptation to divergent selection mediated by resource levels or predation, for instance.

The respiratory burst response also varied significantly among populations. At the population level, the respiratory burst response tended to increase with increasing *D. gasterostei* abundance. Therefore, divergent *Diplostomum*-mediated selection may play a role in shaping investment in this component of the innate immune response. This pattern has been reported for several other host-parasite systems (Lindström *et al.*, 2004; Whiteman *et al.*, 2006; Bryan-Walker *et al.*, 2007; Cornet *et al.*, 2009) and falls in line with predictions that investment in immune components should be optimised according to the risk of parasite exposure (Shudo & Iwasa, 2001; Lee, 2006; Tschirren & Richner, 2006). Again, as with parasite resistance, this may be particularly likely if there is a cost tied to the maintenance of those components (Lochmiller & Deerenberg, 2000; McKean *et al.*, 2008). To correlate respiratory burst response with natural *D. gasterostei* infection levels, data from control and exposed fish were pooled, since across all fish, *D. spathaceum* exposure did not lead to a significant upregulation of the respiratory burst response. However, this approach obscured some interesting within-population differences between treatments, especially in the Chadha Ruaidh population. Exposed fish from this population showed a markedly higher respiratory burst relative to control, uninfected fish. This variation had no apparent biological significance; there was no concomitant decrease in the number of metacercariae that established in the eye lens compared to the other four populations, in which there was no change in the respiratory burst following *D. spathaceum* exposure. Nevertheless, as Chadha Ruaidh suffers the highest burdens of *D. gasterostei* in the wild, it suggests that *Diplostomum*-mediated selection may have shaped the response to infection, at least in this population.

The proportion of granulocytes, the effector cells that mount the respiratory burst response (Dahlgren & Karlsson, 1999; Magnadóttir, 2006), was another important factor explaining variation in the respiratory burst response. The intensity of the respiratory burst was significantly positively correlated with the percentage of granulocytes, even though this measure itself did not vary significantly among populations. Importantly, since the population-level differences in respiratory burst were found after correcting for variation in the proportion of granulocytes, it suggests

these differences were not merely the result of differences in numbers of granulocytes present in a given sample. Instead, there may be qualitative differences in granulocytes among populations, for example in their efficiency at converting oxygen to reactive oxygen species, or 'activation status' (Kurtz *et al.*, 2004; Kalbe & Kurtz, 2006).

The experiment convincingly demonstrated that fish develop an adaptive immune response to *Diplostomum* infection. Twice-exposed fish showed a significant decrease in the number of *D. spathaceum* metacercariae in the second round of exposure following one previous exposure. This appears to be a common feature of *Diplostomum* infections (Höglund & Thuvander, 1990; Karvonen *et al.*, 2005; but see Kalbe & Kurtz, 2006). Interestingly, the effect was present in all populations, even in the population that was the most susceptible and was not naturally exposed to *Diplostomum*, Lochmaddy. However, evidence for presence of antibodies following repeated exposure, a clear sign of the development of an adaptive immune response, is equivocal. For instance, Bortz *et al.* (1984) detected antibodies specific to *Diplostomum* in wild-caught fish, whereas Stables and Chappell (1986a) and Höglund & Thuvander (1990) failed to find any *Diplostomum*-specific antibody in experimentally infected fish. Moreover, Whyte *et al.* (1990) reported that trout immunised with *D. spathaceum* had significantly lower infection levels than controls when fish were artificially infected five weeks post-immunisation, but there was no association between the degree of protection and the level of serum antibody in individual fish. Regardless of the potential role of acquired immunity in the wild (Karvonen *et al.*, 2004a), genetically-based differences in the innate response appear to be more important in determining *Diplostomum* resistance (Rauch *et al.*, 2006a; this study).

I found no growth cost of *D. spathaceum* infection. Most evidence of the detrimental growth effects of *Diplostomum* is anecdotal (Pennycuik, 1971b; Buchmann & Uldal, 1994; Marcogliese *et al.*, 2001b). Only a handful of studies have examined growth costs formally by comparing the growth of exposed and unexposed hosts in a common garden setup. For example, Karvonen & Seppälä (2008) found no overall difference in growth rate between whitefish, *Coregonus lavaretus*, exposed to a large dose of *Diplostomum spathaceum* and those that were unexposed. Only when cataract coverage reached 100% was there a detectable effect on growth (Karvonen & Seppälä,

2008). Likewise, Ballabeni and Ward (1993) observed no effect of *Diplostomum phoxini* infection on minnow growth. In fact, in a follow-up experiment, it was reported that minnows exposed to low doses actually grew faster than either control fish or fish exposed to a high parasite dose (Ballabeni, 1994). As regards the stickleback-*Diplostomum* interaction, a recent field transplant experiment revealed that sticklebacks with higher burdens of *D. gasterostei* sometimes grew faster, but that this effect differed between stickleback populations (MacColl & Chapman, 2010). This study provides further evidence for the minimal impact of *Diplostomum* infection on host growth. It is worth noting that any growth costs associated with *Diplostomum* infection are probably indirect, mediated via the effects of reduced vision on competitive and foraging abilities (Buchmann & Uldal, 1994; Karvonen & Seppälä, 2008). Competitive scenarios can exacerbate the cost of parasitism (Bedhomme *et al.*, 2005). Perhaps I failed to detect a growth cost because of the feeding regime that minimised effects of competitive ability on food intake (fish were provided food to satiation daily). This is certainly the case for infection with another parasite of sticklebacks, *Schistocephalus solidus* (Barber *et al.*, 2008). Nevertheless, the results from this study strongly indicate that there is no growth (energetic) cost of being infected. There was significant variation in growth rate among populations, however. In Chapter 7 I explore whether there is a genetically based trade-off, that is, an evolutionary cost of resistance, between growth and *Diplostomum* resistance (see e.g. Kuukka-Anttila *et al.*, 2010). Costs of resistance form an important mechanism for the maintenance of variation in parasite resistance within and among natural host populations (Sheldon & Verhulst, 1996; Rigby *et al.*, 2002; Summers *et al.*, 2003; Lazzaro & Little, 2009), and as mentioned before, may ultimately contribute to our understanding of the spatial patterns of variation in *D. spathaceum* resistance.

To conclude, I have shown that a measure of the innate immune system, the respiratory burst response, does not accurately reflect resistance to *Diplostomum spathaceum* among stickleback populations. Although the respiratory burst response is known to have a functional role in killing the migrating stages of this parasite species, variation in this immune component could not explain variation in resistance following a single *Diplostomum* exposure. This indicates that it is not sufficient to use just one measure of the immune response as a proxy for parasite resistance. Other innate immune components may be involved in resistance to this parasite that have

yet to be identified. Nevertheless, I found significant variation in resistance to *D. spathaceum* and the respiratory burst response among populations, which probably has a genetic basis, although I cannot rule out maternal effects due to the full-sib experimental design. There was no significant correlation between natural infection of a closely related parasite species, *Diplostomum gasterostei* and resistance to *D. spathaceum*, but the only population that was not naturally exposed to *D. gasterostei* was significantly less resistant to *D. spathaceum*, which may be the result of reduced selection to maintain resistance. However, the respiratory burst response tended to increase with increasing natural *D. gasterostei* abundance. Therefore, although divergent *Diplostomum*-mediated selection, as inferred from natural infection levels, may shape investment in both parasite resistance and the innate immune response to some extent in evolutionarily young stickleback populations, these two aspects are not correlated. Spatial variation in the stickleback-*Diplostomum* interaction and the relationship between parasite resistance and immune response in this system appear to be complex and requires further investigation.

CHAPTER 6: EXAMINING VARIATION IN RESISTANCE AND THE INNATE IMMUNE RESPONSE TO A TAPEWORM AMONG STICKLEBACK POPULATIONS

6.1 Introduction

The dynamics of interspecific interactions are likely to vary in space, as is their importance for shaping the evolution of the interacting species (Thompson, 1999; 2005). Interactions between parasites and their hosts provide particularly good models for investigating the influence of spatial variation on trait divergence (Burdon & Thrall, 1999; Dybdahl & Storfer, 2003; Brockhurst *et al.*, 2004) since many parasites are known to have detrimental effects on the fitness of their hosts and therefore form potent agents of selection (Summers *et al.*, 2003). Host traits that contribute to the outcome of host-parasite relationships, such as parasite resistance, are known to vary spatially. For example, differences in parasite resistance between natural populations of the same host species are commonplace in plants (Kaltz *et al.*, 1999; Thrall *et al.*, 2002; Laine, 2005) and invertebrates (Ebert *et al.*, 1998; Kraaijeveld & Godfray, 1999; Lively & Dybdahl, 2000). A few examples also exist for vertebrates (McCoy *et al.*, 2002; Jackson & Tinsley, 2005). Considering that the immune system forms a crucial mechanism of host defence (Janeway *et al.*, 2004), it is not surprising that geographic variation in immune responses (Sanjayan *et al.*, 1996; Bryan-Walker *et al.*, 2007; Cornet *et al.*, 2009) and in the genes underlying them, such as those of the major histocompatibility complex (Miller *et al.*, 2001; Bernatchez & Landry, 2003; Wegner *et al.*, 2003), are equally pervasive. However, it is not always clear what drives these patterns of variation. One possibility is that investment in parasite resistance and immune response reflects adaptation of host populations to the local abundance/prevalence of individual parasites species. We might expect individuals from populations facing higher risks of parasitism to be under strong selection to evolve defence mechanisms (Schmid-Hempel & Ebert, 2003; Lindström *et al.*, 2004; Corby-Harris & Promislow, 2008). However, very few studies have explicitly examined

the relationship between natural infection levels and parasite resistance, as determined by artificial infection experiments in a laboratory ('common garden') environment. Even if populations are not selected *a priori* on the basis of natural infection levels, it remains important to quantify population-level variation in parasite resistance and immune response, because if such variation has a genetic basis, it provides the first line of evidence for the potential of divergent parasite-mediated selection (Little, 2002).

Here, I exploit the well-established three spined stickleback (*Gasterosteus aculeatus*)-*Schistocephalus solidus* model (Barber & Scharsack, 2010), by carrying out an artificial infection experiment with laboratory-reared sticklebacks from five populations, to explore geographic variation in this host-parasite interaction. *S. solidus* is a common body cavity cestode of sticklebacks that has detrimental effects on many aspects of stickleback fitness, including body condition (Tierney *et al.*, 1996), growth (Barber & Svensson, 2003; Wright *et al.*, 2007; Barber *et al.*, 2008), reproduction (Rushbrook & Barber, 2006; Heins & Baker, 2008) and behaviour (Giles, 1983a; Milinski, 1984; Barber & Huntingford, 1995). The impacts of *S. solidus* infection on stickleback reproduction are particularly severe. Field data have shown that the majority of infected females and males may be unable to produce gametes and sperm (Arme & Owen, 1967; McPhail & Peacock, 1983; Tierney *et al.*, 1996), effectively reducing the host's fitness to zero (Lafferty & Kuris, 2002; Heins & Baker, 2008). In one study, mortality of large numbers of *S. solidus*-infected fish over a short time period was recorded (Pennycuik, 1971c). Accordingly, *S. solidus* is frequently cited as an agent of selection in stickleback populations, and selection on resistance to this parasite is expected to be high (Hammerschmidt & Kurtz, 2005; Scharsack *et al.*, 2007b). It is becoming increasingly clear that there may be spatial variation in the impacts of *S. solidus* parasitism on stickleback fitness. For example, field data from several Alaskan populations indicate that infected female sticklebacks in these populations are still able to produce eggs (Schultz *et al.*, 2006; Heins & Baker, 2008), albeit of a smaller size (Heins & Baker, 2003), in contrast to female sticklebacks in many European populations. Likewise, MacNab *et al.* (2009) reported that wild-caught *S. solidus*-infected males from two populations in England differed in their ability to build nests and court females. These results raise the possibility that populations experience different strengths of *S. solidus*-mediated selection, with a knock-on effect for the

evolution of population-level differences in *S. solidus* resistance and stickleback life history. As yet, there is no study that has examined experimentally variation in resistance to *S. solidus* among multiple stickleback populations.

Recent immunological studies have added an additional perspective to the stickleback-*Schistocephalus* interaction (reviewed by Hammerschmidt & Kurtz, 2009). For instance, Kurtz et al. (2004) found a relationship between major histocompatibility complex (MHC) class IIB diversity and *S. solidus* parasite index (a measure of worm mass in relation to host mass), suggesting that resistance to *S. solidus* may have an immunogenetic basis. It has also been shown that the respiratory burst response, an effector mechanism of the cellular innate immune system mediated by granulocytes such as neutrophils (Dahlgren & Karlsson, 1999; Magnadóttir, 2006), is upregulated in *S. solidus*-infected fish relative to uninfected fish (Kurtz et al., 2004; Scharsack et al., 2004). However, a temporal study of the stickleback immune response to *S. solidus* revealed that upregulation occurs only at a late stage of the infection (Scharsack et al., 2007b), rather than at an early stage of infection when the respiratory burst response may be more effective at eliminating small tapeworms (Secombes & Chappell, 1996; Hammerschmidt & Kurtz, 2007; Hammerschmidt & Kurtz, 2009). Lastly, Wedekind & Little (2004) reported that an immune response activated independently and prior to exposure to *S. solidus* reduced prevalence of *S. solidus* infection by 50%. Taken together, these results highlight that the stickleback immune response may be important in governing the outcome of *S. solidus* infection, although the interaction of *S. solidus* with the stickleback immune system is likely to be complex and highly variable. As for measures of parasite resistance, it is important to assess the immune response to *S. solidus* in a number of populations to evaluate the generality of findings.

Therefore, the aims of the study were to: 1) to assess variation in resistance and the innate immune response to *S. solidus* among populations, 2) examine whether variation in resistance was linked to variation in natural *S. solidus* infection levels, and 3) determine if *S. solidus* infection impairs stickleback growth and energy status. Like the immunological studies of the stickleback-*S. solidus* interaction outlined above, I used the proportion of granulocytes and the respiratory burst response as measures of the innate immune response. The third aim was of interest because, although the

impacts of *S. solidus*-infection on fish growth have been well documented (Barber *et al.*, 2008), the extent to which experimental costs of infection vary among populations has never been assessed.

6.2 Methods

6.2.1 Fish breeding and natural infection levels

Sexually mature male and female sticklebacks were collected from five geographically isolated lochs on North Uist, Outer Hebrides, Scotland in May 2008. Fish were caught using minnow traps (Gee traps, Dynamic Aqua, Vancouver) which were set overnight and lifted the following day. A small sample (approximately 20 fish per loch) from the total caught was dissected to determine natural prevalence and abundance of *Schistocephalus solidus* (Table 6.1). For each population, F_1 offspring used in infection experiments were obtained by making 8 sets of unrelated full-sib crosses (families) from wild-caught fish. To make a cross, eggs were stripped from a gravid female and placed into a Petri dish containing a small volume of 1 ‰ salt solution. Males were killed, by overdose of anaesthetic (400 mg L⁻¹ MS222), and dissected to remove testes. Fine forceps were used to tease apart testes and release sperm, which was gently mixed with the eggs (Barber & Arnott, 2000). Two to three hours later, fertilisation was confirmed by low-power microscopy (separation of membranes), and testes were removed from the fertilised clutches. Fertilised eggs were transferred to a falcon tube containing 50 mL of 1 ‰ salt solution. Eggs were then transported on ice to aquaria at the University of Nottingham, where they were placed in a plastic cup with a mesh screen on the bottom suspended in a well-aerated tank containing dechlorinated water (Marchinko & Schluter, 2007). Water was treated with Methylene blue to reduce the probability of fungal infection. After 10 days, egg cups were transferred to individual half-tank partitions of 100L tanks and the eggs were allowed to hatch. Following hatching, full-sib families were thinned to groups of 15. Clutches from each population were distributed haphazardly between tanks across the temperature-controlled room (13.5°C ± 1°C). Fry were fed with infusoria (*Colpidium* spp.) for the first 5 days, then daily with brine shrimp (*Artemia salina*) naupliae until day 65 post-hatching. Thereafter, fish received chironomid larvae ('bloodworm'; defrosted from

frozen) daily. Fish were maintained at a daylight regime mimicking the natural photoperiod on North Uist.

Population	<i>S. solidus</i> abundance	<i>S. solidus</i> prevalence	N	Geographic location
Chadha Ruaidh	0	0 (0.0, 15.9)	21	57°35"N; 7°11"W
Hosta	0.95 ± 0.29	45.0 (24.4, 68.0)	20	57°37"N; 7°29"W
Lochmaddy	0	0 (0.0, 13.9)	24	57°36"N; 7°10"W
Reivil	0	0 (0.0, 26.5)	11	57°36"N; 7°30"W
Tormasad	0.05 ± 0.05	5.0 (0.3, 24.4)	20	57°33"N; 7°19"W

Table 6.1 The five stickleback populations from North Uist used in the study. Data on *Schistocephalus solidus* prevalence and abundance were obtained by dissecting approximately 20 sticklebacks from each loch in May 2008. Abundance values are reported with the standard error of the mean whereas prevalence values are given with 95% confidence intervals.

6.2.2 Experimental design

The experiment compared two treatments ('exposed' and 'control') among five stickleback populations and was divided into three replicate blocks. Within blocks, seven and three fish per population were assigned to the 'exposed' and 'control' treatments, respectively (50 fish per block; total $n = 150$). Exposed fish were fed an artificially infected copepod, whereas control fish were sham-exposed (fed an uninfected copepod) (Fig. 6.1). The imbalance in sample sizes between treatment groups ensured that a sufficient number of infected fish was obtained for immunological measurement; infection success rates vary enormously among *S. solidus* artificial infection experiments and can be low (see e.g. Arnott *et al.*, 2000; Barber & Svensson, 2003; Kurtz *et al.*, 2004). Control fish were included for growth comparisons with infected fish and to obtain baseline measures of immunological parameters. Due to the lack of statistical power associated with balancing treatments across families within blocks, fish family was used as a controlling factor instead of a balancing factor; that is, for each population, fish from the same 8 full-sib families were included per block. However, since the total sample size per population within

blocks was 10, another fish from each of two randomly-selected families was included. In the case of the Hosta population only 7 full-sib families were available. Fish were randomly assigned to the control or exposure treatment. For logistic purposes, block 3 was separated from the other two blocks by six weeks. Fish were 13-15 months old at the start of the infection experiment and had therefore reached sexual maturity. As a result, sex was balanced across populations where possible. Owing to intrinsic differences in fish lengths among populations (see Chapter 7) fish could not be size-matched across populations. Hence, size variation was controlled for *post hoc* in statistical models.

Fish were dissected at 45-47 days after exposure to determine infection status and to obtain immunological measurements (the proportion of granulocytes in head kidneys and the intensity of the respiratory burst response). This time point was chosen because a recent study showed that sticklebacks upregulate the respiratory burst response at this stage of *S. solidus* infection (Scharsack *et al.*, 2007b). As not all fish became infected following exposure to a single *S. solidus* procercoid, they were divided into three groups: 'infected', 'exposed-uninfected' and 'control'. The number of fish that could be processed for respiratory burst analysis was restricted to 32 per block; therefore, only six fish per population were selected (30 per block). Three infected fish and the three control fish were chosen from each population. If fewer than three fish became infected or control fish had died, randomly selected exposed-not-infected fish were included to make up the number of fish per population to six.

6.2.3 Parasite culture

In order to obtain *S. solidus* plerocercoids for worm breeding, sticklebacks infected with *S. solidus* were caught from the lower pond of Jubilee Campus, University of Nottingham (52°57"N; 1°11"W) using minnow traps. Prevalence and abundance of *S. solidus* in this population remain high throughout the year (personal observation). *S. solidus* was cultured *in vitro* following the technique of Wedekind (1997). Plerocercoids were removed aseptically from fish and transferred to 6.3 mm semi-permeable dialysis tubing (Visking). Each tube was suspended in a 100-250 ml conical flask containing 100ml of sterilised Minimal Essential Medium with Earle's salts and L-glutamine (Sigma-Aldrich), 25mM HEPES-buffer (Sigma-Aldrich), 1 g L⁻¹

penicillin/streptomycin and 6.5 g L⁻¹ D-Glucose. Two large (> 100 mg), size-matched worms from different fish hosts were selected per culture flask. Large worms were chosen because *S. solidus* egg output is proportional to worm size (Wedekind *et al.*, 1998) and larger worms produce more outcrossed eggs (Luscher & Milinski, 2003). Size-matching further increases the probability of outcrossing (Luscher & Milinski, 2003), which in turn increases the hatching rate of eggs (Schjorring, 2004; Milinski, 2006). Each conical flask was wrapped in aluminium foil and placed in an incubator at 40°C, a setup that mimics the conditions inside the gut of the final, bird host. Ninety-six hours post-incubation, worms were removed and the eggs that had collected in the tubing were flushed out with tap water into a glass Petri dish. Eggs were rinsed three times, transferred to small 20 mL universal tubes containing tap water (wrapped in aluminium foil) and stored in a refrigerator at 4°C until use. Twenty-one days prior to hatching, eggs were left at room temperature. One day before infection of copepods, eggs were transferred to a small glass Petri dish and exposed to fluorescent light for 12 hours to induce hatching. Eggs from two *S. solidus* crosses (culture flasks) were used for the experiment.

6.2.4 Copepod infection

Laboratory-reared copepods, *Cyclops strenuus*, were maintained individually in wells of a 24-well ELISA plate containing 2 mL of Chalkley's medium (per litre of deionised water: 20 g NaCl, 0.8 g KCl and 1.2 g CaCl₂). To expose copepods to *S. solidus*, they were fed a single coracidium (tapeworm larva). Thereafter, they were fed three times weekly with *Colpidium* spp. and kept on a 16L:8D cycle at 22°C ± 2°C. The infection status of copepods was determined ten days post-exposure by placing each copepod in a drop of Chalkley's medium and screening them under a high-power microscope (x 40). Copepods were maintained for 15-17 days before being fed to sticklebacks; by this stage procercooids had developed a cercomer, a characteristic feature of infective procercooids (Hammerschmidt & Kurtz, 2009).

6.2.5 Stickleback infection

Three days before exposure to a copepod, individual sticklebacks were measured to the nearest 0.1 mm and transferred from their family tank to 3L plastic tanks containing 1 L of dechlorinated water. During this period fish were starved to increase the probability of copepod consumption. On the day of exposure, each fish was fed one infected copepod. Fish belonging to the ‘control’ treatment were sham-exposed by being fed one uninfected copepod; control fish were handled in an identical manner to ‘exposed’ fish. To determine whether the copepod had been consumed, the water was filtered through a 70 µm cell strainer (BD Falcon). If the copepod was still present, the fish was re-fed the same infected copepod. Fish were re-fed bloodworm once they consumed the infected/uninfected copepod. The majority of fish achieved this in one day (78.6%), although several fish only consumed the copepod two (14.6%) or three (6.8%) days post-exposure. Throughout the experiment, fish were maintained on a 16L:8D cycle and water was changed every four days. Fish were fed daily with frozen bloodworm (defrosted from frozen) at a standardised ration of 8% body weight (*sensu* Barber & Svensson, 2003).

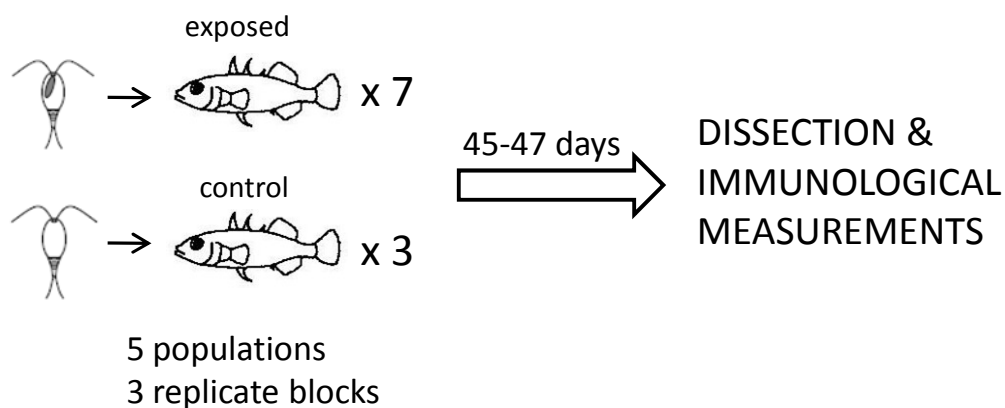


Figure 6.1 The experimental design of the *S. solidus* infection experiment. Exposed fish were fed a copepod infected with a single *S. solidus* procercoid, whereas control fish were fed an uninfected copepod (sham-exposed). Stickleback image from Kalbe & Kurtz (2006) and copepod image from Hammerschmidt & Kurtz (2005).

6.2.6 Dissection and immune measurements

On day 45-47 post-exposure fish were killed, by overdose of MS222 (400 mg L⁻¹) and destruction of the brain, and measured to the nearest 0.1mm as before and weighed to the nearest 1 mg. As some fish did not consume the copepod on the first day, the actual day post-exposure (i.e. post-consumption) varied between 45 days and 47 days. Nevertheless, all fish from the same block were processed on the same day. Fish were dissected and the body cavity was checked for *S. solidus*. Plerocercoids were weighed to the nearest mg. Livers were also dissected out and weighed to the nearest 1 mg. Throughout the experiment, if fish were found in a state of poor health, as defined by reduced feeding activity and cessation of movement, they were euthanised by overdose of MS222 (400 mg L⁻¹) and destruction of the brain. For the purpose of the experiment, this was defined as mortality. If *S. solidus*-infected fish had to be euthanised, infection status was determined immediately after euthanasia, where possible. Otherwise, fish were stored in 70% ethanol until infection status could be ascertained.

As measures of cellular innate immune system, the proportion of granulocytes and the respiratory burst response were quantified. Extraction of head kidney leukocytes (HKL) and quantification of HKL cell numbers followed a protocol modified after Scharsack et al. (2004). Head kidneys were dissected out using fine forceps. HKLs were isolated by squeezing head kidneys through a 40 µm cell strainer (BD-Falcon) into a falcon tube containing 2 mL 'R-90'. R-90 is RPMI-1640 (without phenol red, Gibco) diluted with 10% distilled water, a medium that matches stickleback osmolarity (Scharsack *et al.*, 2004). Cells were washed once by centrifugation at 4°C and resuspended in a final volume of 500 µL R-90. Cells and media were kept on ice throughout the protocol.

HKL numbers were quantified by a standard cell dilution assay. To individual flow cytometry tubes the following were added: 50 µL of washed HKLs, 100 µL R-90, 5 × 10⁴ green fluorescent beads (3 µm, Polyscience) and propidium iodide (2 mg L⁻¹, Sigma-Aldrich). Propidium iodide is a DNA-binding substance that enables the detection of dead cells. Using a flow cytometer (FACS, Becton & Dickinson), FSC/SSC

characteristics of at least 10,000 events were acquired at linear scale; fluorescent intensities at wavelengths of 530 (beads) and 585nm (propidium iodide) were acquired at log scale. Data were analysed using FACS Diva software. Cellular debris was easily distinguished from viable cells by its low FSC values and was excluded from subsequent analyses. Likewise, fluorescent beads (green fluorescence positive) were easily differentiated from viable HKLs (propidium iodide negative, green fluorescence negative) and dead HKLs (propidium iodide positive, green fluorescence negative). The total number of viable HKLs per sample was then calculated as: $N \text{ [HKLs]} = \text{events [viable HKL]} \times \text{number [fluorescent beads]} / \text{events [fluorescent beads]}$. To standardise cell volumes for the respiratory burst assay, HKL suspensions were adjusted to a final concentration of $6.25 \times 10^5 \text{ cells mL}^{-1}$. The flow cytometric data were also used to calculate ratios of granulocytes to lymphocytes, which have characteristically high SSC and low SSC profiles, respectively (Scharsack *et al.*, 2004; Serada *et al.*, 2005).

Respiratory burst activity was measured using an *in vitro* luminol-enhanced chemiluminescence assay. The luminol buffer was prepared by adding luminol dissolved in 0.1M NaOH (10 mg mL^{-1}) to a buffer solution (25 μL of dissolved luminol per ml of Hanks' buffered saline solution (HBSS, Gibco), containing 20mM HEPES (Sigma-Aldrich) and 1 g mL^{-1} bovine serum albumen (BSA, Sigma-Aldrich)). 20 μL of luminol buffer was added to 160 μL of HKL suspension ($10^5 \text{ cells per well}$) in wells of 96-well microtitre plate. To allow the cells to take up luminol, plates were incubated at room temperature for 30 minutes. Release of reactive oxygen species (ROS) was stimulated by the addition of 20 μL of a zymosan suspension (7.5 g L^{-1} phosphate-buffered saline (PBS, Gibco)). When enough cells were available, a duplicate zymosan treatment and a control PBS-only treatment were also included. The respiratory burst reactions were measured at room temperature at three minute intervals for two hours using a microtitre plate luminometer (Berthold Technologies). Relative luminescence (RLU) was tracked using WinGlow software (Berthold Technologies). The amount of light emitted in a given reaction therefore provides a quantitative measure of the number of ROS produced by HKLs in a sample. From each reaction curve, the peak of the respiratory burst response was determined (hereafter 'respiratory burst').

6.2.7 Statistical analysis

6.2.7.1 Natural *S. solidus* infection

All statistical analyses were conducted in GenStat (release 12, VSN International Ltd., Hemel Hempstead, U.K.). Parameter estimates of statistical models are given for significant effects only. A generalised linear model (GLM) with a negative binomial error distribution and a logarithm link function was used to analyse variation in natural abundance of *S. solidus*. Population was the only fixed effect in this model.

6.2.7.2 Mortality in the infection experiment

Mortality in the artificial infection experiment was analysed with a generalised linear mixed model (GLMM). ‘Died?’, the response variable, was modelled with a binomial error distribution and a logit link function. Block was included as a random effect, whereas sex, length before exposure (‘initial length’), population, infection status (‘infection’) and infection \times population were included as fixed effects. Likelihood ratio (LR) tests, a common method for assessing significance of random effects in mixed models (Galwey, 2006), are not possible in GLMMs with one random effect. Therefore, an informal significance test was carried out that compares the variance component estimate with its standard error; if the standard error exceeded the estimate, the random effect was considered non-significant and dropped from the model (*sensu* Galwey, 2006). In this case, the model reverted to a GLM. Stepwise model simplification was used to obtain a minimum adequate model (MAM) by sequential deletion of non-significant fixed effects (Crawley, 2007). Significance of fixed effects was assessed by noting the change in deviance following the deletion of the effect and comparing it to a χ^2 distribution with the appropriate degrees of freedom. If main effects were marginal to interaction effects, significance of main effects was tested in the presence of interactions.

6.2.7.3 Experimental *S. solidus* prevalence and size, and the relationship between natural *S. solidus* infection and experimental *S. solidus* prevalence

Prevalence of *S. solidus* infection in the artificial infection experiment was modelled with a GLMM. Prevalence was interpreted to be inversely proportional to *S. solidus* resistance. Infection success ('Infected?') was the binary response variable, taking a value of 1 if the fish was infected and a value of 0 if it was uninfected, and was fitted with a binomial distribution and logit link function. The fixed effects were population, sex, initial length, initial length \times population and sex \times population. Block was included as a random effect. The significance of fixed and random effects was assessed as above. A general linear model was used to analyse variation in *S. solidus* plerocercoid weight. The response variable, parasite index (PI), a measure of worm weight relative to fish body weight, was calculated using the equation (Arme & Owen, 1967):

$$PI = (\text{plerocercoid weight} / \text{fish WWG}) * 100$$

where WWG represents weight without gonads. WWG was used instead of total body weight as some females had reached reproductive maturity and had developed egg clutches, which can account for a large proportion of total body weight. WWG did not exclude parasite weights due to their negligible contribution to overall host body weight (see results). PI was log-transformed to achieve normality. Due to the small number of infected fish, the model included only population as a fixed effect. Pearson's correlation analysis was used to determine the relationship at the population level between mean natural *S. solidus* abundance and experimental *S. solidus* prevalence (*S. solidus* resistance); a two-tailed test was used to assess the significance of this correlation.

6.2.7.4 Immune measures

Linear mixed models (LMM) were used to analyse immunological data. The first model analysed variation in the proportion of granulocytes ('prop gran' as the response variable). The fixed model consisted of the effects sex, length at time of dissection ('length'), treatment, population and treatment \times population. A second model was used to analyse variation in the intensity of the respiratory burst response. 'Respiratory burst', square root-transformed to achieve normality, was the response variable. Sex, length, treatment, population, prop gran and treatment \times population were included as fixed effects. Respiratory burst data were missing for four fish as a result of insufficient numbers of HKLs and two fish were excluded from the model due to very low respiratory burst values. Block was included as a random effect. The significance of the block effect was assessed using a LR test, by comparing the difference in deviances of the reduced model (without the random effect) and the full model to a χ^2 distribution with one degree of freedom. This was possible because LMMs, in contrast to GLMMs, do not require the specification of a random model. If block was not significant, the model reverted to a general linear model. As above, a backward procedure was used for model selection with the sequential deletion of non-significant effects until a MAM was specified. Significance of fixed effects was assessed by Wald F tests.

6.2.7.5 Fish growth and condition indices

To examine the effects of *S. solidus* infection on fish growth a LMM was used, with specific growth rate (SGR; *sensu* Barber, 2005) of individual fish as the response variable. SGR, defined as the average daily percentage increase in length, was calculated using the equation:

$$\text{SGR} = 100 * [\ln (L_{t1}/L_{t0})]/(t_1 - t_0)$$

where L_{t1} and L_{t0} represent the standard length measured before, and at the end of, the experimental infection, respectively, and $t_1 - t_0$ denotes the number of days between measurements. This was 46 for blocks 1 and 3 and 47 for block 2. Block was fitted as a

random effect. The fixed model included the effects: sex, initial length, infection, population and infection \times population. ‘Infection’ comprised only two levels: infected (‘i’) and uninfected (‘o’), the latter of which also included exposed-uninfected fish. One fish was excluded from this analysis because it had a negative SGR and another was excluded as its initial length was not measured. Additionally, the impact of *S. solidus* infection on two measures of energy status, the hepatosomatic index (HSI) and the body condition factor (K), was investigated. HSI is a measure of medium-term energy reserves (Chellappa *et al.*, 1995) and was calculated using the equation:

$$\text{HSI} = (\text{W}_{\text{liver}}/\text{WWG}) * 100,$$

where W_{liver} represents liver weight. HSI was square-root transformed to achieve normality. K was calculated using the equation (Bolger & Connolly, 1989):

$$\text{K} = (\text{WWG}/\text{L}^3) * 100,000$$

In both the HSI and K LMMs, sex, population, infection and infection \times population were included as fixed effects, and block was included as a random effect. Significance of fixed and random effects was assessed as described for immune measures.

6.3 Results

6.3.1 Natural *S. solidus* infection

There was significant variation among populations in natural abundance of *S. solidus* ($\chi^2_4 = 11.07$, $P < 0.001$; Table 6.1). *S. solidus* was absent from Chadha Ruaidh, Lochmaddy and Reivil.

6.3.2 Mortality in the infection experiment

In total, 22 fish died during the experiment. Mortality did not vary significantly among blocks (variance component estimate \pm S.E. = 0.37 ± 0.59). Initial length ($\chi^2_1 = 0.81$, $P = 0.368$), population ($\chi^2_4 = 0.45$, $P = 0.775$), *S. solidus* infection ($\chi^2_1 = 0.96$, $P = 0.328$) and

the interaction between infection and population ($\chi^2_4 = 0.65$, $P = 0.625$) did not explain significant variation in mortality. There was a non-significant trend for males to die at a higher rate ($\chi^2_1 = 2.99$, $P = 0.084$),

6.3.3 Experimental *S. solidus* prevalence and size, and the relationship between natural *S. solidus* infection and experimental *S. solidus* prevalence

Due to the shortage of successfully infected copepods, two fish from Tormasad in the exposed treatment were not fed an infected copepod. As a result, only 100 exposed fish were included in the analysis of *S. solidus* prevalence. Infection success rate in the artificial infection experiment was low: 15 out of the 100 fish exposed to a singly-infected copepod became infected (15%). *S. solidus* prevalence did not differ significantly among blocks (variance component estimate \pm S.E. = 0 ± 0); therefore, data from all blocks were pooled and the model reverted to a GLM. Prevalence of *S. solidus* varied significantly among populations (Table 6.2, Fig. 6.2a). Prevalence was dependent on initial length, with larger fish more likely to become infected than smaller fish (Table 6.2). However, the significant interaction between initial length and population indicates that the effect of length was dependent on population (Table 6.2). In fact, the length \times population interaction was strongly influenced by one data point: a small, infected fish from Lochmaddy. Removal of this data point altered the output of the GLM substantially: initial length \times population ($\chi^2_4 = 7.00$, $P = 0.136$) and population ($\chi^2_4 = 6.18$, $P = 0.180$) became non-significant, although initial length remained significant ($\chi^2_1 = 5.96$, $P = 0.015$). Sex and sex \times population failed to explain significant variation in *S. solidus* prevalence (Table 6.2). Overall, the worms that were recovered were very small (mean weight \pm S.E. = 2.9 ± 0.6 mg). Parasite index (PI), a measure of worm weight relative to fish weight, did not vary significantly among populations ($F_{4,10} = 0.85$, $P = 0.524$; Fig. 6.2b). At the population level, experimental *S. solidus* prevalence was negatively correlated with mean natural *S. solidus* abundance but the relationship was not significant ($r = -0.55$, $P = 0.340$; Fig. 6.3). Hosta and Tormasad, the two populations in which *S. solidus* was present, had the lowest prevalence of *S. solidus* in the infection experiment.

Fixed effect	χ^2	df	P	Estimate \pm S.E.
Population	17.63	4	0.024	
Chadha Ruaidh				-2.576 \pm 1.178
Hosta				-2.639 \pm 1.178
Lochmaddy				-0.985 \pm 0.508
Reivil				-1.189 \pm 0.697
Tormasad				-12.964 \pm 31.907
Initial length	14.29	1	0.014	0.143 \pm 0.076
Sex	1.72	1	0.190	-
Initial length \times Population	10.58	4	0.032	
Chadha Ruaidh \times Initial length				0.438 \pm 0.245
Hosta \times Initial length				-0.087 \pm 0.331
Lochmaddy \times Initial length				-0.071 \pm 0.121
Reivil \times Initial length				0.064 \pm 0.167
Tormasad \times Initial length				2.210 \pm 5.800
Sex \times Population	2.46	4	0.652	-

Table 6.2 Results from the GLM of *S. solidus* prevalence in the infection experiment. The response variable, infection success (the likelihood of becoming infected), was modelled with a binomial distribution and a logit link function.

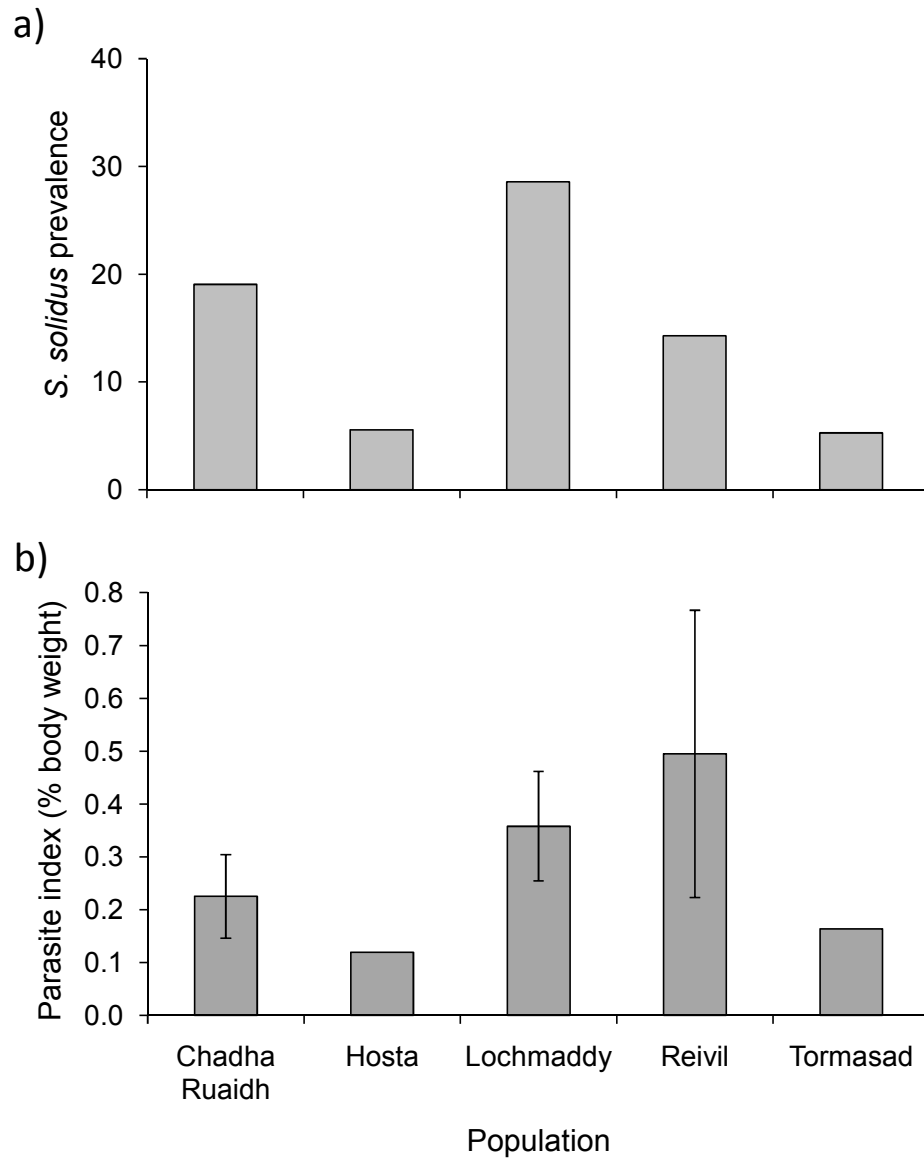


Figure 6.2 a) Variation in the prevalence of *S. solidus* among the five stickleback populations in the infection experiment. Fish were exposed to a copepod infected with one *S. solidus* procercoid and dissected 47 days later to determine infection status. Sample sizes for all populations are 21, with the exception Tormasad (19 exposed fish) and Hosta (18 exposed fish). Parasite prevalence was interpreted to be inversely proportional to resistance to *S. solidus*. b) Population variation in *S. solidus* parasite index (PI), a measure of worm weight relative to the fish's weight. PI (% body weight) was calculated using the equation: $PI = 100 \times (\text{worm weight} / \text{fish weight including worm weight})$. Error bars are the standard error of the mean.

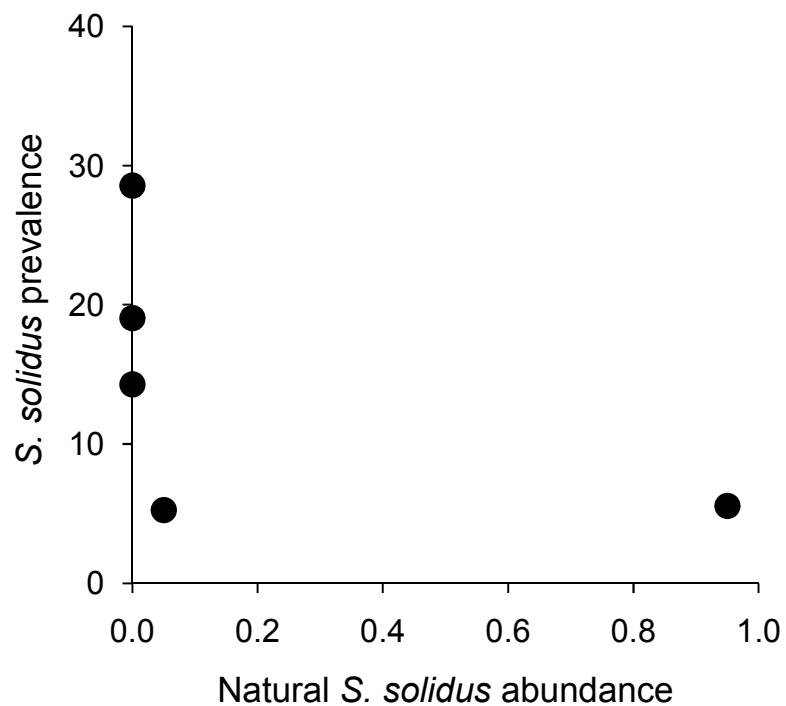


Figure 6.3 Relationship at the population level between *S. solidus* prevalence in the infection experiment and mean *S. solidus* abundance in the wild (measured in May 2008): $r = -0.55$, $P = 0.340$.

6.3.4 Immune measures

The proportion of granulocytes differed significantly between blocks (LR test: $\chi^2_1 = 2.78$, $P = 0.048$, variance component estimate \pm S.E = 0.0013 ± 0.0017). The proportion of granulocytes varied significantly among populations ($F_{4, 71.5} = 2.78$, $P = 0.034$), but this effect was dependent on treatment (treatment \times population: $F_{8, 71.5} = 2.83$, $P = 0.009$, Fig. 6.4b). However, there was no overall significant difference in the proportion of granulocytes between *S.solidus*-infected fish, exposed-uninfected fish and control fish ($F_{2, 71.7} = 2.71$, $P = 0.073$). Variation in the proportion of granulocytes was not associated with length ($F_{1, 71.1} = 0.00$, $P = 0.971$) nor sex ($F_{1, 69.8} = 0.00$, $P = 0.998$). Respiratory burst did not vary significantly among blocks (LR test: $\chi^2_1 = 0.02$, $P = 0.444$, variance component \pm S.E. = 0 ± 0); therefore data from all three blocks were pooled. There was no significant difference between the respiratory burst of *S.solidus*-infected fish, exposed-uninfected fish and control fish ($F_{2, 74} = 0.56$, $P = 0.574$), and this was consistent across populations (treatment \times population: $F_{8, 64} = 1.06$, $P = 0.399$; Fig. 6.4b). However, respiratory burst varied significantly among populations ($F_{4, 76} = 5.08$, $P = 0.001$; Fig. 6.4b). Respiratory burst was positively and significantly correlated with the proportion of granulocytes ($F_{1, 76} = 10.42$, $P = 0.002$; parameter estimate \pm S.E. = 189.1 ± 58.6). Sex ($F_{1, 72} = 0.00$, $P = 0.984$) and length ($F_{1, 73} = 0.05$, $P = 0.820$) did not have a significant effect on respiratory burst.

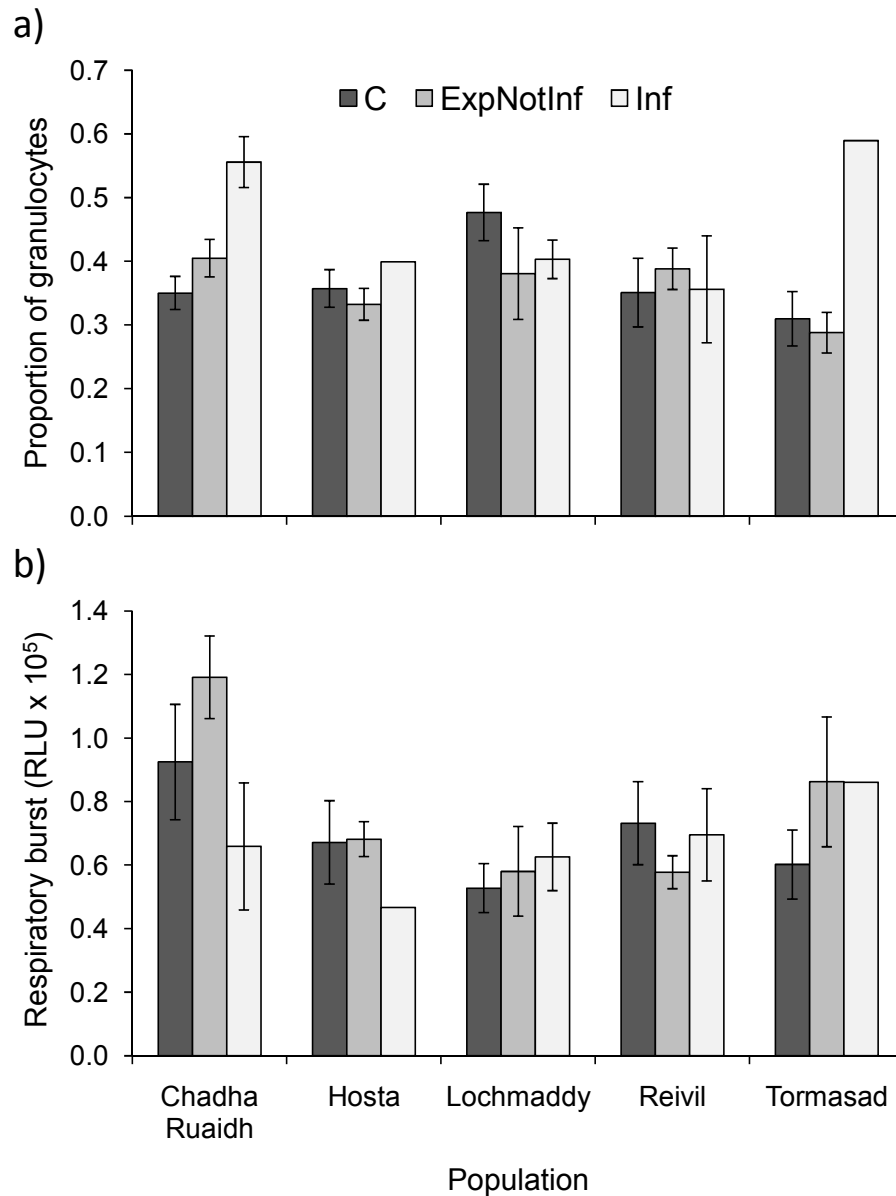


Figure 6.4 Inter-population variation in measures of the innate immune response in the *S. solidus* infection experiment: a) proportion of granulocytes and b) respiratory burst response. Measures of immune parameters were obtained 45-47 days after feeding fish one *S. solidus*-infected or uninfected copepod. The proportion of granulocytes was determined by flow cytometry, whereas measures of the respiratory burst response were obtained using an *in vitro* luminol-enhanced chemiluminescence assay. 'C', 'ExpNotInf' and 'Inf' denote control, exposed-uninfected and infected fish, respectively. Error bars are the standard error of the mean.

6.3.5 Fish growth and condition

Variation in specific growth rate (SGR) among blocks was not significant (LR test: $\chi^2_1 = 0.00$, $P \gg 0.05$, variance component estimate \pm S.E. = 0 ± 0); hence data from all blocks were pooled. There was no significant difference in SGR between *S. solidus*-infected fish and uninfected fish ($F_{1, 116} = 2.57$, $P = 0.111$; Fig. 6.5a) and this was similar across populations (infection \times population: $F_{4, 111} = 0.75$, $P = 0.561$). SGR was dependent on population ($F_{4, 117} = 3.9$, $P = 0.005$; Fig. 6.5a) and was negatively correlated with initial length ($F_{1, 117} = 5.03$, $P = 0.027$; parameter estimate \pm S.E. = -0.0029 ± 0.0015). Males and females did not differ significantly in SGR ($F_{1, 115} = 0.16$, $P = 0.690$).

The hepatosomatic index (HSI) did not vary significantly among blocks (LR test: $\chi^2_1 = 2.40$, $P = 0.061$, variance component estimate \pm S.E. = 0.0034 ± 0.0047). *S. solidus* infection did not affect HSI ($F_{1, 118} = 0.01$, $P = 0.923$; Fig. 6.5b) and this was consistent across populations (infection \times population: $F_{4, 114} = 1.54$, $P = 0.195$). HSI varied significantly among populations ($F_{4, 119} = 21.62$, $P < 0.001$; Fig. 6.5b) and between males and females ($F_{1, 119} = 40.70$, $P < 0.001$). Females had a higher HSI than males (parameter estimate \pm S.E.: males = 3.51 ± 0.15 ; females = 4.55 ± 0.15). There was no significant difference in body condition factor (K) among blocks (LR test: $\chi^2_1 = 0.15$, $P = 0.35$, variance component estimate \pm S.E. = 0 ± 0). K did not differ significantly between *S. solidus*-infected fish and uninfected fish ($F_{1, 118} = 0.65$, $P = 0.422$; Fig. 6.5c), and this was the same in all populations (infection \times population: $F_{4, 114} = 1.30$, $P = 0.275$). Population ($F_{4, 119} = 19.65$, $P < 0.001$; Fig. 6.5c) and sex ($F_{1, 119} = 31.62$, $P < 0.001$) explained significant variation in K. Males had a higher K than females (parameter estimate \pm S.E.: males = 1.01 ± 0.02 ; females = 0.88 ± 0.02).

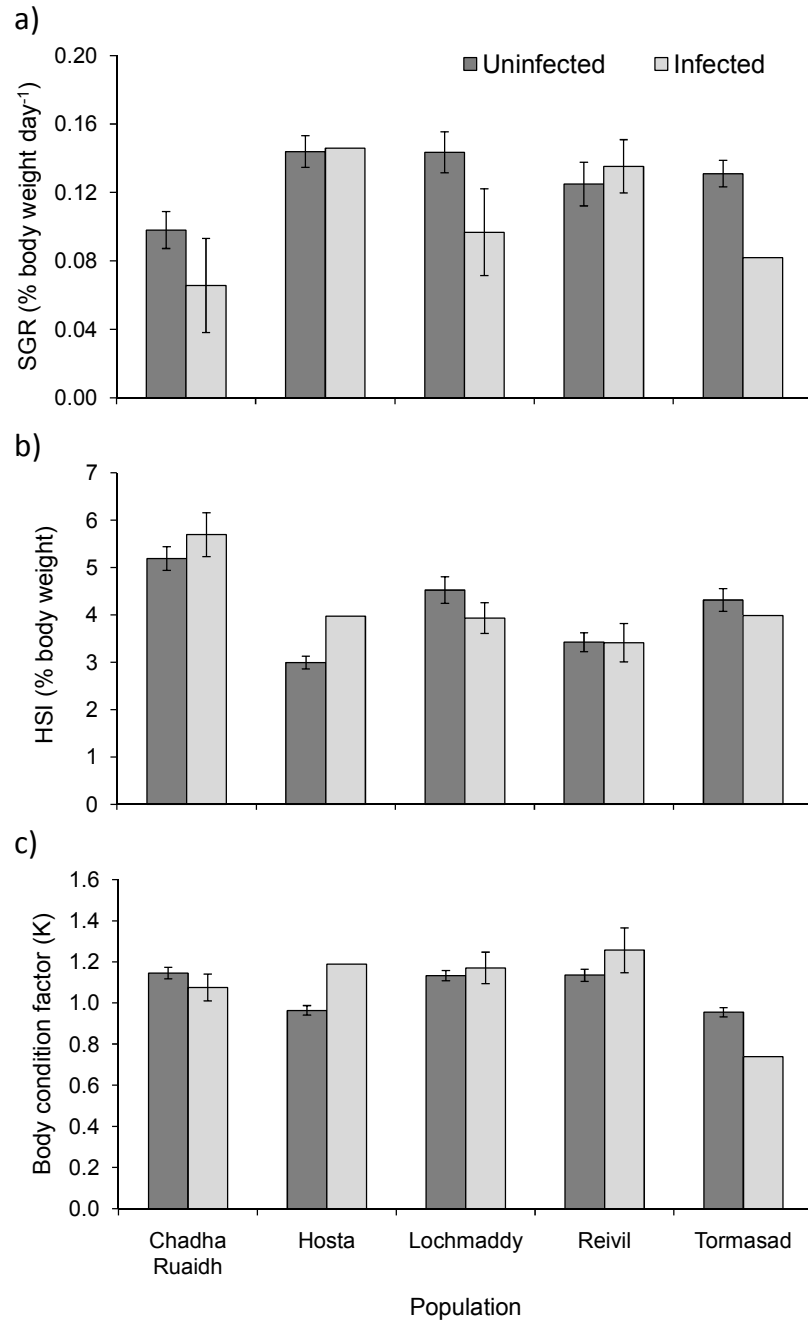


Figure 6.5 Differences in specific growth rate (SGR), hepatosomatic index (HSI) and body condition factor (K) between *S. solidus*-infected fish and uninfected fish across populations. Specific growth rate, a measure of the average daily percentage increase in fish length, was calculated using the equation: $SGR = 100 * (\ln(L_{t1}/L_{t0}) / (t_1 - t_0))$, where L_{t1} and L_{t0} represent the standard length measure before, and at the end of, the experimental infection and $t_1 - t_0$ denotes the number of days between measurements (45-47). The hepatosomatic index was calculated using the equation: $HSI = (\text{liver weight} / \text{fish weight without gonads (WWG)}) * 100$, whereas body condition factor (K) was calculated as: $K = (\text{WWG} / \text{standard length}^3) * 100000$. Error bars are the standard error of the mean.

6.4 Discussion

The infection experiment revealed that there was minor but significant variation in the prevalence of *S. solidus* among stickleback populations. As *S. solidus* prevalence was defined to be (inversely) proportional to resistance, it suggests that these populations vary in resistance to *S. solidus*. Previous infection experiments have documented variation in the response to *S. solidus* exposure among families of the same population (e.g. Arnott *et al.*, 2000), but to my knowledge, this is the first study to examine variation in resistance among multiple stickleback populations. Nevertheless, the low infection success rate (just 15% of exposed hosts became infected) warrants a cautious interpretation of this result. The effect of population in the statistical (GLM) model of *S. solidus* prevalence was highly dependent on its interaction with fish length, measured before fish were exposed. Overall, larger fish were more likely to become infected, but this pattern was not identical across populations. The model was sensitive to the addition of a single data point: a small, infected Lochmaddy fish that generated the negative relationship between length and infection in this population, and also the interaction between length and population. Exclusion of this data point changed the model output substantially, such that *S. solidus* prevalence did not vary significantly among populations. Therefore, the five populations probably do not vary in resistance to *S. solidus*. The low infection success rate is comparable to other *S. solidus* infection experiments that have exposed fish to a single proceroid (e.g. Barber & Svensson, 2003: 16.7%). Failure of *S. solidus* to establish may stem from either variation in host response (resistance) or from variation in viability of proceroids (infectivity). Given that low infection rates were common to all populations, the latter provides a more suitable explanation.

Like *S. solidus* prevalence, the *S. solidus* parasite index (PI), a measure of the severity of infection (Arme & Owen, 1967), did not vary significantly among populations. In fact, growth of plerocercoids over the 47-day period was remarkably limited. In most fish, worms attained a final size of less than 3mg, far from the 50mg threshold weight required to become infective to the final bird host (Tierney & Crompton, 1992). In a comparable experiment, Scharsack *et al.* (2007b) recorded an average plerocercoid weight of just over 50mg at day 47 post-infection. The small worm size, combined

with the low infection success rate, suggests that the strain of *S. solidus* used in the experiment may have been avirulent. Use of a local North Uist strain of *S. solidus* rather than a strain derived from a population where prevalence is high throughout the year (>70%, personal observation) may present a very different picture of spatial variation in resistance to *S. solidus*. It has been suggested that PI reflects resistance to *S. solidus* (Kurtz *et al.*, 2004), but this seems incompatible with what we know about the *S. solidus* infection process (Hammerschmidt & Kurtz, 2009). Although knowledge of the mechanisms conferring resistance to *S. solidus* is limited, histological and immunological studies indicate that tapeworms are probably destroyed only at an early stage of infection, before they become established in the body cavity (Hammerschmidt & Kurtz, 2007; Scharsack *et al.*, 2007b). Moreover, Wedekind & Little (2004) found that resistance to *S. solidus* is potentially an ‘all-or-nothing’ (qualitative) response. Therefore, resistance is probably best defined by host infection status (infected vs. uninfected) rather than by growth of the worm once it has established in the body cavity. The ability of the host to limit growth of *S. solidus* in the body cavity, which minimises the fitness costs of infection, may instead reflect a tolerance response (Corby-Harris *et al.*, 2007; Raberg *et al.*, 2007; Boots, 2008), a concept which has not yet been explored for the stickleback-*S. solidus* interaction.

There was no relationship between abundance of *S. solidus* in the wild and *S. solidus* resistance in the infection experiment. A weak pattern in the data was that the three populations where *S. solidus* was absent showed higher infection levels than the two populations where *S. solidus* was present. Studies on other host-parasite systems have recorded a similar pattern (Kalbe & Kurtz, 2006; Bryan-Walker *et al.*, 2007; Hasu *et al.*, 2009), as have infection experiments I have carried out with two other macroparasite species in the same five populations (Chapter 4 and 5). Arguably, these two populations are more susceptible to *S. solidus* infection because there has been less selection to maintain resistance (Webster *et al.*, 2004; Lohse *et al.*, 2006), particularly if maintaining resistance carries an evolutionary cost (Sheldon & Verhulst, 1996; Rigby *et al.*, 2002). However, as Hasu *et al.* (2009) have pointed out, it is difficult to establish reliably that a population is (historically) unexposed to the parasite. This applies especially to macroparasites that are usually distributed in an aggregated manner within host populations: a few individuals carry a disproportionately large parasite burden relative to the rest of the population (Shaw *et al.*, 1998; Wilson *et al.*, 2002).

Given that the sample size per loch was small and that prevalence of *S. solidus* in North Uist is generally low (Chapter 3), the abundance recorded may not be entirely representative of natural infection levels. For example, although *S. solidus* appeared to be absent from Chadha Ruaidh in 2008, more extensive sampling from this loch in 2009 revealed that *S. solidus* was actually present at a very low level. Furthermore, *S. solidus* abundance and prevalence are known to vary seasonally (Pennycuik, 1971c). This may explain why no pattern was found.

The respiratory burst response varied significantly among populations. This component of the innate immune system is known to play an important part in the cellular immune response to fish helminths (Whyte *et al.*, 1989; Taylor & Hoole, 1995; Secombes & Chappell, 1996), and has previously been shown to be upregulated in *S. solidus*-infected fish relative to sham-exposed control fish (Kurtz *et al.*, 2004; Scharsack *et al.*, 2004; Scharsack *et al.*, 2007b). Here, I observed no such change, and surprisingly, this was common to all populations. This argues against population-differences in response to *S. solidus* infection. As emphasised in the methods, the 47-day interval was chosen on the basis of an infection experiment by Scharsack *et al.* (2007b), in which the temporal profile of the stickleback immune response was monitored. The study demonstrated that upregulation of the respiratory burst response was greatest on this day post-infection (Scharsack *et al.*, 2007b). However, it is questionable whether mounting a respiratory burst response at a late stage of a *S. solidus* infection is functionally relevant (Hammerschmidt & Kurtz, 2009). Small worms, either in the gut or migrating through the intestinal wall to the body cavity, presumably are more vulnerable to attack by the reactive oxygen species produced by a respiratory burst response than large, well-established worms (Read & Skorping, 1995; Secombes & Chappell, 1996; Scharsack *et al.*, 2007b). As explained above, it is predominantly at this early stage of an infection that procercoids fail to establish (Hammerschmidt & Kurtz, 2007). Therefore, measuring the respiratory burst response immediately after exposure rather than at day 47 post-infection may increase the likelihood of capturing relevant variation in the immune response to *S. solidus* among populations (but see Scharsack *et al.*, 2007b). I acknowledge that this is a limitation of the current study, but argue that it is difficult to determine infection status accurately when tapeworms are small; therefore, the choice of day 47 post-infection was a

compromise between obtaining reliable information about parasite establishment and activation of the innate immune response.

The proportion of granulocytes had a strong impact on, and was positively correlated with, the intensity of the respiratory burst response. Granulocytes are a group of white blood cells that include neutrophils, the main effector cells of the respiratory burst response (Secombes, 1996; Dahlgren & Karlsson, 1999). Therefore, it is not surprising that quantitative differences in the proportion of granulocytes account for a considerable proportion of variation in the respiratory burst response. *S. solidus* infection did not increase the proportion of granulocytes, in contrast to other studies that have demonstrated a mobilisation of granulocytes following *S. solidus* infection (Kurtz *et al.*, 2004; Scharsack *et al.*, 2004). However, there was a significant interaction between population and treatment for the granulocyte data, hinting at the possibility of a population-specific immune response to *S. solidus* infection. Due to the small number of infected fish per population this finding should be interpreted with caution, as in the case of the statistical model of *S. solidus* resistance. Moreover, if upregulation of the innate immune response is linked to the size of the tapeworm, the small worm sizes at day 47 may have hindered the ability to detect significant changes in immune parameters.

I found no evidence for a cost of *S. solidus* infection in terms of growth or energy status indices, neither within nor across populations. The impacts of *S. solidus* infection on stickleback growth have received considerable attention and are known to be strongly affected by rearing conditions (Barber *et al.*, 2008). In the current study, fish were maintained on a standardised ration of 8% body weight, a feeding regime which has been associated with decreases in fish growth following *S. solidus* infection (Barber & Svensson, 2003). A possible explanation for the lack of an observed effect is that tapeworms were too small to impair growth. As with impairment of reproduction, the effects of *S. solidus* on growth may be a side-product of the infection resulting from nutrient theft (Schultz *et al.*, 2006; Heins & Baker, 2008), rather than an adaptive host or parasite life history strategy (Minchella, 1985). Presumably, nutrient demands increase proportionately with size of the *S. solidus* plerocercoid, and the small worms may have put little, if any, strain on stickleback nutrient allocation. The indices of energy status, the hepatosomatic index and the

body condition factor, followed a very similar pattern to growth rates, and the same explanation may hold for the lack of an effect of *S. solidus* infection on these measures. It seems that only restricted group feeding conditions or short-term food deprivation lead to a negative effect of *S. solidus* infection on growth rate and energy indices, at least in a laboratory environment (Barber *et al.*, 2008). In fact, *ad libitum* feeding conditions may even increase stickleback growth (Arnott *et al.*, 2000). Although there were no differences in the effects of parasitism on growth and energy status among populations, there was ample population-level variation in all three measures, indicating considerable life history differences between the five stickleback populations. Ultimately, such variation could play a role in determining the outcome of *S. solidus* infection, both in the lab and in the wild. For example, Barber *et al.* (2005) showed that *S. solidus* grows faster in faster-growing sticklebacks. Therefore, the impacts of *S. solidus* on reproduction, and stickleback biology generally, may be exacerbated in populations where fish grow faster.

In conclusion, I did not find significant variation in resistance to *S. solidus* among the five stickleback populations, as inferred from variation in *S. solidus* prevalence in the artificial infection experiment. The infection success rate was very low, which may be attributable to the low viability (infectivity) of the parasites from the source population. Resistance was not correlated with natural *S. solidus* abundance, indicating that population variation in resistance may not be the result of adaptation to the local rate of parasite exposure, which was assumed to reflect divergent parasite-mediated selection. The proportion of granulocytes and the respiratory burst response showed substantial population-level variation, but neither innate immune parameter was upregulated following *S. solidus* infection. There was also no effect of *S. solidus* infection on growth or energy indices in any population. However, the ability to detect changes in immune, growth and energy measures was probably limited by the extremely slow growth of *S. solidus* worms inside the host, and the associated minimal impact on host fitness. A repeat of the experiment, involving a local and potentially more virulent North Uist parasite strain as well as a larger host sample size, is required to clarify spatial patterns of *S. solidus* resistance and immune response to infection in this system.

CHAPTER 7: ARE THERE TRADE-OFFS BETWEEN JUVENILE GROWTH RATE AND PARASITE RESISTANCE OR THE INNATE IMMUNE RESPONSE IN STICKLEBACKS?

7.1 Introduction

Animal populations in the wild are characterised by genetic variation in parasite resistance (Henter & Via, 1995; Ebert *et al.*, 1998; Paterson *et al.*, 1998; Lambrechts *et al.*, 2005; Chapters 4-5) and immune response (Mucklow *et al.*, 2004; Cichon *et al.*, 2006; Cotter *et al.*, 2004; Chapters 5-6). It is relatively straightforward to demonstrate that such variation exists, by conducting infection experiments and immune assays with outbred, lab-reared individuals from natural populations. However, demonstrating how this variation is maintained has proven to be a much more difficult task. Costs of resistance and immune response constitute one potential mechanism maintaining variation in these traits (Sheldon & Verhulst, 1996; Lochmiller & Deerenberg, 2000; Rigby *et al.*, 2002; Zuk & Stoehr, 2002; Schmid-Hempel, 2003). Investment in parasite resistance may be traded-off with investment in other traits. The concept of trade-offs is a central paradigm in life history theory and is widely invoked to explain the maintenance of genetic variation in fitness traits (Price & Schluter, 1991; Houle, 1992; Stearns, 1992). However, it is only recently that parasite resistance and immune responses have been integrated into this framework (Sheldon & Verhulst, 1996; Lochmiller & Deerenberg, 2000; Rigby *et al.*, 2002; Zuk & Stoehr, 2002; Schmid-Hempel, 2003). Given a finite amount of resources, an organism faces competing demands for these resources from different life history traits (Zera & Harshman, 2001). Over time, trade-offs mediated by physiology become hard-wired, leading to genetic correlations between life history traits, some of which may be negative (antagonistic pleiotropy) (Stearns, 1989; Roff, 1992). Since not all traits can be maximised by natural selection, trade-offs constrain the evolution of life histories (Reznick *et al.*, 2000; Roff & Fairbairn, 2007).

There are several well-established examples in the literature of trade-offs between parasite resistance and other life history traits. For example, Kraaijeveld & Godfray (1997) found a trade-off between larval competitive ability and resistance to the parasitoid *Asobara tabida* in *Drosophila melanogaster*. Likewise, Webster & Woolhouse (1999) reported that *Biomphalaria glabrata* snails that were more resistant to the trematode *Schistosoma mansoni* suffered reduced fecundity in the absence of parasitism. Trade-offs between particular immune components, rather than resistance phenotypes, and other life history traits appear to be more pervasive. Numerous studies have documented costs associated with immune responses in terms of survival (Moret & Schmid-Hempel, 2000; Armitage *et al.*, 2003; Hanssen *et al.*, 2004; Eraud *et al.*, 2009) and reproduction (Adamo *et al.*, 2001; Bonneaud *et al.*, 2003; French *et al.*, 2007; McKean *et al.*, 2008). However, it is necessary to distinguish two different types of immune costs: those associated with maintenance of immune machinery (costs of immune maintenance) and those associated with mounting an immune response (costs of immune activation) (Lochmiller & Deerenberg, 2000; Schmid-Hempel, 2003; McKean *et al.*, 2008). Costs of immune activation typically have a physiological basis and are highly plastic, whereas costs of immune maintenance are partly the product of resource allocation over evolutionary time scales, and are thus more likely to have a genetic basis (Schmid-Hempel, 2003; Cornet *et al.*, 2009). Importantly, the magnitude of the cost is strongly dependent on the type of cost (Shudo & Iwasa, 2001; Armitage *et al.*, 2003; Lee, 2006; Hamilton *et al.*, 2008). The vast majority of work in this area has focussed on costs of immune activation, presumably because of the ease with which such studies can be conducted in natural field populations. Detecting costs of immune maintenance has proven more difficult (Lochmiller & Deerenberg, 2000). Although there are a number of potential trade-offs between life history traits and immune components and parasite resistance, costs of investment in immune components and parasite resistance cannot be assumed to be correlated. This is particularly true if the parameter(s) of the immune system that is measured does not accurately reflect parasite resistance (Keil *et al.*, 2001; Adamo, 2004; Schwarzenbach & Ward, 2007, Chapter 5). Therefore, both costs of immune response and costs of parasite resistance should be examined simultaneously.

A life history trade-off that has received little attention thus far in the context of ecological immunology is the trade-off between juvenile growth and parasite

resistance or immune response (but see e.g. Barber *et al.*, 2001; Coltman *et al.*, 2001). It is widely recognised that animal growth rate is an adaptive, heritable life history trait that is shaped by natural selection (Case, 1978; Arendt, 1997). In fish, juvenile growth rate forms an important component of fitness since faster growth leads to higher overwinter survival, a larger body size, and hence increased fecundity (Wootton, 1984; Conover & Present, 1990; Schluter, 1995; Brown *et al.*, 1998). Furthermore, rapid growth allows fish to attain a size-refuge from predation when predators are gape-limited (Reimchen, 1991; Abrams *et al.*, 1996). Therefore, juvenile growth rate may be a good proxy for fitness (Schluter, 1995; Munch & Conover, 2003). However, growth rates are rarely maximised in natural populations. Instead, growth rates are often locally adapted to environmental conditions (Niewiarowski & Roosenburg, 1993; Schultz *et al.*, 1996; Jensen *et al.*, 2000; Arendt & Reznick, 2005). This implies that there may be costs and constraints associated with the evolution of rapid growth rate. A number of potential trade-offs have been highlighted to attempt to explain the extensive variation in growth rate within and among populations (Arendt, 1997); many of these invoke proximate mechanisms, mediated by behaviour or physiology. For instance, faster growth is normally associated with increased foraging effort which in turn increases the risk of mortality through predation (Werner & Anholt, 1993; Stoks *et al.*, 2005; Biro *et al.*, 2006).

Rapid growth may also incur evolutionary costs. For example, in *Drosophila* there is a negative genetic correlation between growth rate and larval survival (Chippindale *et al.*, 1997) and in the speckled wood butterfly growth rate is traded off with starvation endurance (Gotthard *et al.*, 1994). With regards to an evolutionary trade-offs between growth and parasite resistance, most evidence for fish comes from the aquaculture industry; the main aim of fish breeding is to select increased body size without a concomitant decrease in resistance to disease (Gjedrem, 2000). Although some studies report a negative genetic correlation between growth and parasite resistance (Henryon *et al.*, 2002; Silverstein *et al.*, 2009), a positive correlation has also been documented (Rye *et al.*, 1990; Gjedrem *et al.*, 1991; Imsland *et al.*, 2002). Data on this trade-off in natural populations are more limited, but the few studies that have been conducted suggest that the sign of the relationship may be equally variable. For example, Barber *et al.* (2001) documented a trade-off between juvenile growth and resistance to the body cavity tapeworm *Schistocephalus solidus* in a population of

three-spined sticklebacks, whereas Coltman et al. (2001) found a positive genetic correlation between growth and resistance to strongylid nematodes in a population of Soay sheep. Genetic correlations between growth and parameters of the immune system are also unpredictable (Koella & Boete, 2002; Cotter *et al.*, 2004; Seppälä & Jokela, 2010) and may depend on the immune components that are measured. Therefore, more studies are required, particularly in vertebrate organisms, that investigate phenotypic and genetic relationships between growth and parasite resistance and growth and the immune response, to determine potential evolutionary costs of investment in these life history traits.

Here, I carried out a growth experiment to quantify variation in juvenile growth rates in five populations of the three-spined stickleback, *Gasterosteus aculeatus*, from North Uist, Scotland, and, using data collected previously from the same five populations, examined whether there are trade-offs at the full-sib family level across populations between juvenile growth rate, an innate immune component (the respiratory burst response) and resistance to two common stickleback macroparasites: *Diplostomum spathaceum* and *Gyrodactylus gasterostei*. The three-spined stickleback forms a good model for studying variation in growth rate because of the rapid evolution of population-level differences in body size over short evolutionary time scales (Bell & Foster, 1994). In the North Uist system, fish from different populations in close geographic proximity can exhibit up to 10-fold differences in adult body size (A. MacColl, unpublished observation). Moreover, previous studies have established that variation in growth rates among stickleback populations has a genetic component (Snyder & Dingle, 1989; Wright *et al.*, 2004) and that growth may be traded off against other fitness-related traits (Schluter, 1995; Barber *et al.*, 2001; Robinson & Wardrop, 2002; Barrett *et al.*, 2009). The three-spined stickleback is also an amenable model for investigating population divergence in parasite resistance, due to the established protocols for carrying out artificial infection experiments (see Chapter 4 and 5). The two macroparasite species were chosen because of their detrimental effects on stickleback fitness and their putative roles as agents of selection (Brassard *et al.*, 1982; Owen *et al.*, 1993; Bakke *et al.*, 2007). The infection experiments carried out previously (Chapters 4 and 5) showed that host populations that are naturally unexposed to a parasite species exhibit higher susceptibility in infection experiments than populations that are naturally exposed to the parasite. As these

unexposed populations do not develop resistance (or lose it), it implies that there is a cost associated with maintaining resistance. Given that natural selection on parasite resistance, the immune response and growth is likely to be strong in stickleback populations, a trade-off between these fitness-related traits may arise. In addition to investigating trade-offs between growth and parasite resistance or the immune response, I tested for a trade-off between resistance to the two parasite species. To my knowledge, this is the first study to examine co-variation of experimental resistance to different macroparasite species across natural vertebrate populations.

7.2 Methods

7.2.1 Fish breeding

Fish were collected from five geographically isolated lochs on North Uist, Outer Hebrides, Scotland during May 2008. These lochs were chosen to represent a range of natural parasite infection levels in order to examine spatial variation in parasite resistance (see Chapter 4 and 5). Fish were caught using minnow traps (Gee traps, Dynamic Aqua, Vancouver) which were set overnight and lifted the following day. For each population, F_1 offspring were obtained by making 9 unrelated full-sib crosses (families) from wild-caught fish. To make a cross, eggs were stripped from a gravid female and placed into a petri dish containing a small volume of 1 ‰ salt solution. Males were killed, by overdose of anaesthetic (400 mg L⁻¹ MS222), and were dissected to remove testes. Fine forceps were used to tease apart testes and release sperm, which was gently mixed with the eggs (Barber & Arnott, 2000). Two hours after incubation, fertilisation was confirmed by low-power microscopy and testes were removed from the fertilised clutches. Fertilised eggs were transferred to a falcon tube containing 50 mL of 1 ‰ salt solution. Eggs were then transported on ice to aquaria at the University of Nottingham, where they were placed in a plastic cup with a mesh screen on the bottom, suspended in a well-aerated tank containing dechlorinated water (Marchinko & Schluter, 2007). Water was treated with Methylene blue to reduce the possibility of fungal infection. After 10 days, egg cups were transferred to individual half-tank partitions of 100L tanks and the eggs were allowed to hatch. Following hatching, full-sib families were thinned to groups of 15. Clutches from each population were

distributed haphazardly between tanks across the temperature-controlled room ($13.5^{\circ}\text{C} \pm 1^{\circ}\text{C}$).

7.2.1 Growth experiment

All 9 sib-families per population were included in the growth experiment, with the exception of Hosta and Reivil, for which only 8 families were available due to high mortality in the remaining family. Throughout the experiment, fish were maintained under a 16L:8D photoperiod. Fry were fed daily with infusoria (*Colpidium* spp.) for the first five days, then twice daily with brine shrimp (*Artemia salina*) naupliae until day 64 post-hatching. Thereafter, fish received chironomid larvae ('bloodworm'; defrosted from frozen) daily. Food was provided to excess.

Starting on day five post-hatching, fry were digitally photographed (using a Nikon D80 camera) at set intervals, following a method modified from Wright et al. (2004). Digital photography provides a valuable tool for tracking growth of young fry as it minimises the stress associated with handling of fish, to which fry are particularly sensitive. Individual fry were transferred from their family tank to a small glass dish (60mm diameter) containing water to a standard depth of 20 mm and were illuminated from above by fluorescent lighting. A 1-mm laminated calibration grid was placed underneath the glass dish to enable calibration of the analysis software, Adobe Photoshop (Adobe Systems, Mountain View, CA). Photographs were taken immediately after fry adopted a resting position (straightened body and tail) on the bottom of the dish, and standard lengths were calculated in Photoshop. Photographing every fish per full-sib families was not possible; therefore, five fish from each family were haphazardly selected for measurement at each time point. The time points were 5, 8, 15, 22, 27, 37, 43, 50, 64, 85, 106, and 127 days post-hatching.

7.2.3 Measures of parasite resistance and the innate immune response

To establish whether there is variation in parasite resistance among North Uist stickleback populations, artificial infection experiments were conducted with two common macroparasites of sticklebacks: the monogenean *Gyrodactylus gasterostei* and the digenean trematode *Diplostomum spathaceum*. Fish from 8 full sub-families

per population (7 for Hosta) were included in the infection experiments; where possible, the same families were used to those in the growth experiment. The protocols for the infection experiments are described in greater detail elsewhere (Chapter 4 and 5), and will only be touched upon briefly here. To examine variation in resistance to *G. gasterostei*, a fin ectoparasite of freshwater sticklebacks, 10-month old fish from each population were infected with two worms, and growth of parasite populations on individual fish was monitored over a period of 62 days. The *Gyrodactylus* infection process is characterised by a standard sequence of events: the parasite population grows, reaches a peak, then starts to decline and is eventually cleared by the host (Bakke *et al.*, 2007). To obtain a general descriptor of resistance to *G. gasterostei*, a principal components analysis was conducted on five parameters of each infection response profile: the peak of the infection, the total worm burden (AUC), average growth rate of the parasite population (r) to the peak, the time taken until peak was reached, and time until the infection was lost following the peak. The scores of principal component 1 (PC1; determined mostly by the peak and AUC) from this analysis served as a proxy for, and were interpreted to be inversely proportional to, *G. gasterostei* resistance: lower PC1 scores corresponded to greater resistance (Chapter 4). For each full-sib family, a mean PC1 score was calculated.

Resistance to *D. spathaceum*, an eye fluke of sticklebacks, was quantified by exposing individual 6-8 month old fish to a standard dose of 20 cercariae, and counting the number of metacercariae that established in the eye lenses (Chapter 5). The infection experiment comprised two exposure rounds, the first approximately six weeks prior to dissection, and the second two days prior to dissection. One group of fish was exposed twice, whereas another group was exposed just once, in the second round of exposure. Resistance to *D. spathaceum* was defined to be inversely proportional to the number of metacercariae in the lenses of fish after a single parasite exposure: the lower the number of metacercariae, the greater the resistance. Data from the two treatments (singly-exposed and doubly-exposed) were pooled to increase the sample size when calculating the mean number of metacercariae for each full-sib family. In addition to the two exposure treatments, a sham-exposed control group was included in the experiment. The respiratory burst response, a powerful effector mechanism of the cellular innate immune response (Dahlgren & Karlsson, 1999), was quantified in fish in all three treatment groups on the day of dissection, to obtain a measure of the innate

immune response. This was achieved by means of an *in vitro* luminol-enhanced chemiluminescence assay on leukocytes extracted from the head kidneys of individual fish (Chapter 5). As there was no significant upregulation of the respiratory burst response following *D. spathaceum* exposure, data from all three treatments were pooled to calculate the mean respiratory burst response per full-sib family. To simplify the interpretation of their relationship to juvenile growth rate and to each other, measures of parasite infection from both infection experiments will be referred to as measures of susceptibility rather than measures of resistance. Therefore, PC1 score in the *G. gasterostei* infection experiment corresponds to susceptibility to *G. gasterostei* whereas the number of metacercariae in the *D. spathaceum* infection experiment corresponds to susceptibility to *D. spathaceum*.

7.2.4 Statistical analysis

All statistical analyses were carried out in GenStat (release 12, VSN International Ltd., Hemel Hempstead, U.K.). Growth trajectories were analysed with a random coefficients model, a form of linear mixed model that is commonly used for repeated measures data sets (Brown & Prescott, 1999). An advantage of a random coefficients model is that it plots a separate regression line for each unit of replication; by setting both the unit of replication and each time point, nested with the unit of replication, as random effects, the model allows the intercept and the slope of each regression line to vary simultaneously. Random coefficients models are particularly useful when the repeated measures are not evenly spaced in time (Brown & Prescott, 1999), as was the case here. In the growth study, fish family was the unit of replication and day was the time point. Day was treated as a continuous variable rather than as a categorical variable. To account for non-independence of fish families, family was nested within population (population \times family). Likewise, the non-independence of repeated measurements of individuals from the same fish family was accounted for by nesting day within family within population, to form the random effect population \times family \times day. Significance of the population \times family \times day random effect was assessed using a likelihood ratio test. Due to marginality, the significance of the population \times family effect could only be assessed informally by comparing the variance component estimate with its standard error; if the standard error was greater than the estimate,

the term was considered non-significant and dropped from the model (*sensu* Galwey, 2006).

Standard length (SL) was the response variable. Although the distribution of SLs was heavily right-skewed, this was the result of the large number of measurements taken at the start of the growth period and the increasing distance between measurement time points towards the end of the experiment. Furthermore, the residuals of the LMM were approximately normally distributed. Therefore, SL, as the response variable, was not transformed. Population, day, initial length (the average length for each fish family recorded on day 5, the first day of measurement), population \times initial length and population \times day were included as fixed effects in the model. Due to mortality in some full-sib families, 'family size' and its interaction with population (family size \times population) were also included as fixed effects. I predicted that fish in smaller families will be disproportionately larger than fish in larger families, since stickleback growth is known to be strongly density-dependent (Wootton, 1984). To obtain a minimum adequate model, non-significant fixed effects were dropped from the model in a stepwise manner (Crawley, 2007). Significance of fixed effects was assessed using Wald F tests. If a fixed effect was marginal to an interaction effect, significance of this effect was tested in the presence of the interaction by fitting it as the last main effect in the model.

To evaluate the relationships between juvenile growth rate, respiratory burst and susceptibility to *G. gasterostei* and *D. spathaceum*, Pearson correlation was performed on family means of these traits (Lynch & Walsh, 1998). This is a standard method for detecting the existence of genetic correlations between life history traits when data have not been collected from the same individuals or from the same experiment (Via, 1984; Roff, 1997). Strictly speaking, this study examined phenotypic correlations, not genetic correlations between stickleback life history traits, which would require a full-sib/half-sib breeding design (Lynch & Walsh, 1998). Nevertheless, in many cases phenotypic correlations are a good approximation for genetic correlations (Roff, 1995; Roff, 1996). To obtain a family-level estimate of growth, the specific growth rate (SGR; *sensu* Barber, 2005), a measure of the percentage daily increase in length, was calculated using the equation: $SGR = 100 * [\ln(L_{122}) - \ln(L_0)] / 122$, where L_{122} and L_0

denote the mean standard length at the end and the beginning of the growth study, respectively. SGR could not be calculated for two Hosta families, as length measurement of individuals from these families commenced on day 27 post-hatching only. Due to the varying levels of mortality across families and the resulting variation in family sizes, it was not possible to obtain measures of parasite resistance and immune response for exactly the same families that were included in the growth study. Therefore, the number families included in a correlation analysis was dependent on the pairwise combination of traits.

7.3 Results

7.3.1 Growth study

The random effects population \times family (variance component estimate \pm S.E. = 0.26 ± 0.07) and population \times family \times day (LR test: $\chi^2 = 75.69$; $P < 0.001$, variance component estimate \pm S.E. = 0.42 ± 0.03) were significant, indicating that there was substantial variation among fish families in both the intercept and the slope of the growth trajectories. Standard length was highly dependent on population, day and the interaction between population and day (Table 7.1; Fig. 7.1). Family size and its interaction with population also had a significant effect on standard length (Table 7.1). However, standard length was not significantly influenced by initial length, and this was consistent across populations (Table 7.1).

7.3.2 Relationship between juvenile growth rate, parasite susceptibility and the innate immune response

There was a significant negative correlation between juvenile growth rate and *D. spathaceum* susceptibility ($r = -0.42$, $n = 36$, $p = 0.011$; Fig. 7.2a). There was no significant correlation between *D. spathaceum* susceptibility and *G. gasterostei* susceptibility ($r = -0.08$, $n = 34$, $p = 0.638$; Fig. 7.2b), between juvenile growth rate and *G. gasterostei* susceptibility ($r = 0.12$, $n = 33$, $p = 0.504$; Fig. 7.2c), nor between juvenile growth rate and respiratory burst response ($r = -0.19$, $n = 37$, $p = 0.261$; Fig. 7.2d).

Fixed effect	F	df	P
Family size	139.74	1, 498.7	<0.001
Population	6.03	4, 36.0	<0.001
Day	9830.60	1, 54.3	<0.001
Initial length	0.02	1, 34.4	0.888
Population \times Day	6.51	4, 55.5	<0.001
Population \times Family size	9.91	4, 428.0	<0.001
Population \times Initial length	2.19	1, 30.1	0.094

Table 7.1 Results from a random coefficient model of standard length (SL) measured at set intervals during the 122-day growth experiment. Population \times family and population \times family \times day were included as random effects.

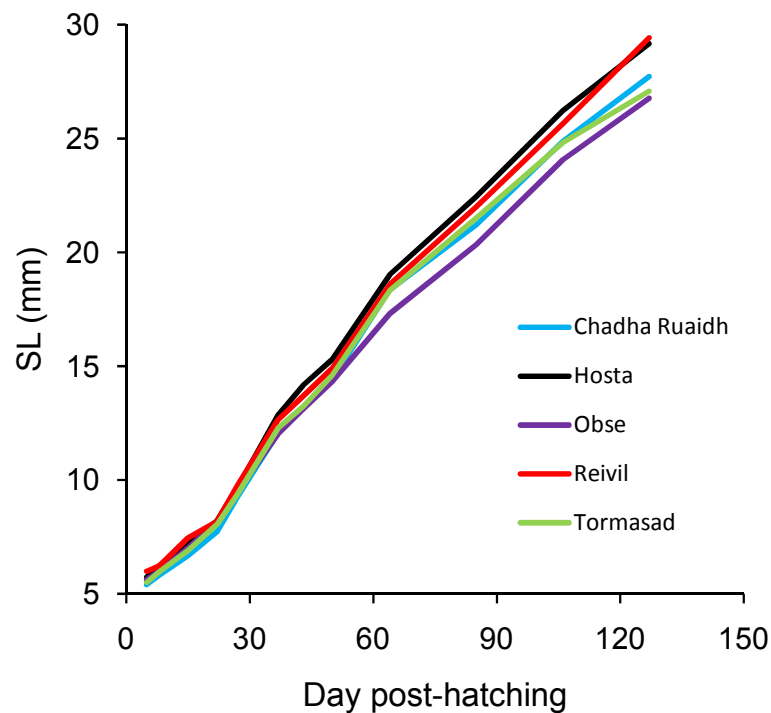


Figure 7.1 Growth trajectories of the five populations over the 122-day growth experiment. Fry from 9 full-sib families per population (8 for Hosta and Reivil) were digitally photographed at set intervals to determine standard length (SL). The line is the population mean. Fish were fed brine shrimp twice-daily until day 64 post-hatching; thereafter, they received bloodworm, defrosted from frozen, daily.

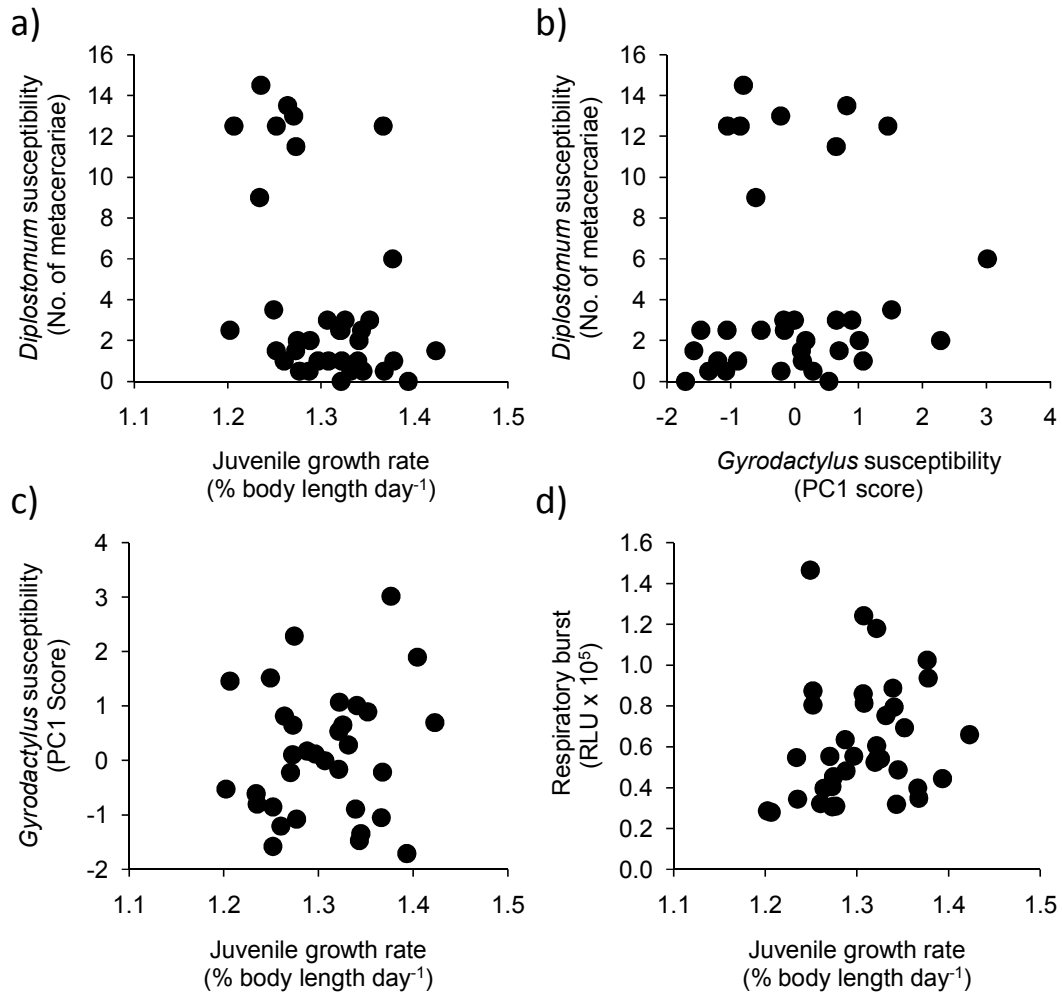


Figure 7.2 Relationships between family means of life history traits: a) juvenile growth rate and *Diplostomum spathaceum* susceptibility ($r = -0.42$, $p = 0.011$), b) *Gyrodactylus gasterostei* susceptibility and *D. spathaceum* susceptibility ($r = -0.08$, $p = 0.638$), c) juvenile growth rate and *G. gasterostei* susceptibility ($r = 0.12$, $p = 0.504$), and d) juvenile growth rate and respiratory burst ($r = -0.19$, $p = 0.261$). Juvenile growth rate was the specific growth rate (SGR) during the growth study and was calculated using the equation: $SGR = 100 * [\ln(L_{122}) - \ln(L_0)] / 122$, where L_{122} and L_0 denote the mean standard length at the end the beginning of the growth study, respectively. Resistance to *G. gasterostei* and *D. spathaceum* were determined from artificial infection experiments. The respiratory burst response, a measure of the innate immune system, was quantified during the *D. spathaceum* infection experiment.

7.4 Discussion

I found significant variation in juvenile growth rate among the five populations of sticklebacks. By the end of the growth study, there was a 2.5mm (9.3%) difference in average length of fry from the slowest and fastest growing populations. These differences only became apparent in the latter half of the study, when mean growth trajectories starting diverging more noticeably. This study supports other common garden experiments in sticklebacks that have also reported considerable levels of spatial variation in juvenile growth rate (Snyder & Dingle, 1989; Wright *et al.*, 2004; Barrett *et al.*, 2009). The common garden conditions indicate that the observed variation has a genetic component. Moreover, since differences in initial length had no significant effect on the growth trajectories, this suggests that variation in juvenile growth rate is independent of maternal effects (mediated via egg provisioning) which are known to can strongly influence the expression of this trait (Einum & Fleming, 1999; Laugen *et al.*, 2002; McAdam & Boutin, 2003). The physiological mechanisms behind the differences in juvenile growth rate have yet to be determined. Both higher digestive performance (Nicieza *et al.*, 1994) and higher food conversion efficiency (Present & Conover, 1992; Imsland *et al.*, 2000) in faster growing fish may play a role.

Growth rate constitutes an important life history trait in juvenile fish because faster growth increases the likelihood of overwinter survival (Conover & Present, 1990; Sogard, 1997; Brown *et al.*, 1998), and is therefore expected to be under strong natural selection. The differences among the five stickleback populations in this study may be the result of divergent natural selection, probably mediated by resource availability or predation regime or both (Arendt & Reznick, 2005). Typically, slow growth is associated with low resource levels (Winemiller & Rose, 1992; Niewiarowski & Roosenburg, 1993; Arendt, 1997). I have yet to quantify spatial variation in resource availability and its relation to fish growth and body size in stickleback populations on North Uist, but preliminary data indicate that there is considerable variation among lochs in densities of zooplankton such as copepods and *Daphnia* (A. MacColl, unpublished data), which form a major component of stickleback diets. Theory predicts that predation selects for rapid growth when prey can attain a size-refuge (Abrams *et al.*, 1996). Given that the two dominant predatory fish on North Uist, the

European eel, *Anguilla anguilla*, and the brown trout, *Salmo trutta*, are gape-limited, different predation regimes among the five populations may select for different juvenile growth rates. Field and experimental studies have shown that predation is a major contributor to population divergence of sticklebacks in terms of body size (McPhail, 1977; Reimchen, 1991; Marchinko, 2009). Again, I have not quantified spatial variation in the intensity of predation in the study populations, but records of the North Uist Angling Club suggest that both trout densities and trout size vary substantially among lochs (A. MacColl, unpublished data).

Other than spatial variation in predator-mediated selection or resource availability, costs associated with rapid juvenile growth may maintain genetic variation in this life history trait. Here, I tested for an evolutionary (genetic) cost associated with fast growth rate. However, I found no evidence for trade-offs at the full-sib family level across populations between juvenile growth rate and resistance to two macroparasite species and the respiratory burst response, a parameter of the cellular innate immune response, in spite of the significant variation in all four traits within and among populations. In fact, I found a significant *negative* correlation between juvenile growth rate and susceptibility to *Diplostomum spathaceum*, indicating that fish families that grow faster are also more resistant to this parasite species. This relationship was due mostly to one population, Lochmaddy, which had a substantially higher susceptibility to *D. spathaceum* than the other four populations. The lack of a trade-off between *Diplostomum* resistance and growth rate corroborates findings from a recent quantitative genetic study in a population of farmed rainbow trout: juvenile fish with more severe cataracts (a measure of susceptibility to *Diplostomum*) tended to have a smaller body size, although the relationship was not significant (Kuukka-Anttila *et al.*, 2010). Therefore, positive covariation with growth rate may be a general feature of resistance to *Diplostomum*.

Only the correlation between juvenile growth rate and *G. gasterostei* susceptibility was positive, hinting at the possibility of a trade-off between juvenile growth rate and *G. gasterostei* resistance, but this relationship was weak and non-significant. Cable & van Oosterhout (2007a) provided some indirect evidence for the importance of body size in mediating the host response to *Gyrodactylus* infection in guppies. They showed that larger guppies developed larger infections of *Gyrodactylus turnbulli*

(Cable & van Oosterhout, 2007a). Regardless of these putative phenotypic correlations (see also Chapter 4), I found no evidence for a genetic trade off between growth rate and *G. gasterostei* resistance. There are just two previous studies of which I am aware that have examined genetic correlations between host growth rate and parasite resistance in natural host populations. Coltman et al. (2001) found a positive correlation between nematode resistance and growth in a wild population of Soay sheep, whereas Barber et al. (2001) reported a negative genetic correlation between juvenile growth rate and resistance to *Schistocephalus solidus* in a lab-reared stickleback population. Therefore, the sign of the relationship appears to be variable and specific to the host-parasite system. Although a trade-off between growth rate and components of the immune response have been demonstrated in some invertebrate species (Koella & Boete, 2002; Cotter *et al.*, 2004; Rantala & Roff, 2005), I did not find a significant genetic correlation between juvenile growth rate and the respiratory burst response across stickleback populations. Thus, it seems that juvenile growth rate does not constrain the evolution of resistance to macroparasites and the immune response in North Uist stickleback populations. Furthermore, there was no significant genetic correlation between susceptibility to *D. spathaceum* and *G. gasterostei*.

There are a number of possible general explanations for why no trade offs were detected. First, fitness costs of parasite resistance depend heavily on the defence mechanisms involved (Rigby *et al.*, 2002; Moret, 2003; Coustau & Théron, 2004). With regards to the immune response, non-specific and constitutively expressed immune defences are thought to be less costly to maintain than specific and inducible defences (Shudo & Iwasa, 2001; Armitage *et al.*, 2003; Lee, 2006; but see Hamilton *et al.*, 2008). This may apply to the partly constitutive, non-specific measure of immune defence used in this study, the respiratory burst response, which was not traded off with juvenile growth rate. There may be other non-specific components of the innate immune response, such as lysozyme or complement activity, or specific ones such as natural antibodies which may prove to be more costly in terms of juvenile growth rate. For example, Mauck et al. (2005) documented a negative correlation between constitutive expression of natural antibodies and growth of nestlings of Leach's Storm Petrel. Although I detected no evolutionary cost of maintaining the respiratory burst, physiological costs of mounting a respiratory burst response are potentially severe.

Specifically, immunopathology resulting from the release of large numbers of reactive oxygen species can contribute to cell damage and ageing (Finkel & Holbrook, 2000; Kurtz *et al.*, 2004). This type of physiological cost was not considered in this study and warrants further investigation. The immunological mechanisms used to achieve resistance to *G. gasterostei* and *D. spathaceum* are currently unknown, but probably differ and are possibly controlled by different genes, given the dramatic differences in infection dynamics of the two parasite species (Chapter 4 and 5). This could explain not only the difference in the relationship between juvenile growth rate and resistance to *D. spathaceum* and *G. gasterostei*, but also the lack of a trade-off between resistance to both parasite species. Indeed, the degree of genetic correlation between traits is assumed to be proportional to the extent of genetic overlap between control of those traits (Schlichting & Pigliucci, 1998) and shared metabolic pathways (de Laguerie *et al.*, 1991).

Second, the defence mechanism(s) conferring resistance may be similar between populations, but populations exposed to higher levels of parasitism may have evolved more efficient defence; that is, the success of defence per unit allocation is higher (Jokela *et al.*, 2000). Accordingly, the costs of parasite resistance and the immune response may vary among populations. The approach adopted here, correlating traits at the full-sib family level across populations, is likely to obscure putative differences among populations in the *within*-population relationship between traits. Third, environmental conditions strongly influence the expression of life history traits and affect the ability to detect genetic relationships between them (Stearns, 1992; Reznick *et al.*, 2000; Sgro & Hoffmann, 2004). Generally, life history traits are more likely to be detected in resource-limited environments than in benign, resource-rich ones (Reznick *et al.*, 2000; Sgro & Hoffmann, 2004). As fish were fed to satiation in all experiments, the high resource conditions may have hindered the ability to detect trade-offs. Furthermore, the environmental conditions were not identical across experiments. For example, during the growth study and *D. spathaceum* infection experiment fish were housed in groups, whereas fish were housed individually during the *G. gasterostei* infection experiment, which may indirectly have influenced the expression of parasite resistance. Fourth, there may a trade-off between parasite resistance or the immune response and a life history trait other than juvenile growth

rate that I did not measure, such as investment in reproduction or competitive ability (Pease & Bull, 1988; Kokko, 1998; Roff & Fairbairn, 2007).

Fifth, identifying correlations between traits from genotypes that represent standing genetic variation in the wild, the approach adopted in the current study, may be difficult (Lazzaro & Little, 2009). Most evidence for genetic trade-offs between parasite resistance and other life history traits comes from selection experiments (e.g. Boots & Begon, 1993; Kraaijeveld & Godfray, 1997; Webster & Woolhouse, 1999; Luong *et al.*, 2007). However, artificial selection is likely to capture mutations of large effect rather than those of small effect, which may occur relatively infrequently in natural populations. Therefore, these experiments potentially overstate the importance and biological relevance of any observed trade-offs (Lazzaro & Little, 2009). Lastly, this study may have lacked the statistical power to detect trade-offs at the full-sib family level: family means were calculated from only 2-3 individuals per family. Although the data are not presented here, there was some within-family variation in all four traits (Chapter 4 and 5). As a result, there is likely to be a large margin of error in these mean values. Nevertheless, this study sheds some light onto the (lack of) genetic trade-offs between juvenile growth rate, parasite resistance and the innate immune response. Quantitative genetic experiments employing a full-sib/half-sib design are required to elucidate the genetic architecture of these traits in these five stickleback populations and to confirm the negative results reported here.

To conclude, I have demonstrated that there is significant variation in juvenile growth rate among North Uist stickleback populations, which has a genetic basis. Integrating data from artificial infection experiments conducted previously, I found no evidence for genetic trade-offs, at the level of full-sib families across populations, between juvenile growth rate and resistance to two common stickleback macroparasite (*D. spathaceum* and *G. gasterosteii*) or a component of the innate immune response (the respiratory burst response), in spite of substantial variation in all four traits among populations. Likewise, there is no evidence for a trade-off between resistance to both parasite species. Thus it appears that these traits do not constrain each other's evolution. The selective factors driving the variation in juvenile growth rate among populations have yet to be determined, but divergent predation regimes and resource availability may play a role.

CHAPTER 8: GENERAL DISCUSSION

In this thesis I used the three-spined stickleback as a model organism to explore spatial variation in host-parasite interactions and the consequences for host population divergence. Here, I re-iterate my main findings, highlight limitations and suggest some avenues for further work.

First, I conducted a field survey of twelve stickleback populations over two years to examine spatiotemporal variation in macroparasite community composition in the study system, North Uist (Chapter 3). I found considerable differences among populations in both the overall diversity and richness of the parasite community and the abundance and prevalence of individual parasite species. This spatial variation was remarkably stable over the two year period, such that the relative differences among populations hardly changed. Assuming that differences in parasite community composition correspond to differences in parasite-mediated selection, this suggests that stickleback populations on North Uist may experience divergent parasite-mediated selection that is consistent in time, with potentially important consequences for the evolution of host traits such as parasite resistance and the immune response.

To examine geographic variation in parasite resistance and immune response, I carried out a series of infection experiments in outbred, lab-reared fish from five stickleback populations with three common stickleback macroparasites: the fin monogenean *Gyrodactylus gasterostei* (Chapter 4), the eye fluke *Diplostomum spathaceum* (Chapter 5) and the body cavity tapeworm *Schistocephalus solidus* (Chapter 6). These parasite species were chosen because they form strong candidates for exerting parasite-mediated selection. Populations differed significantly in resistance to *G. gasterostei* and *D. spathaceum*, but not in resistance to *S. solidus*. In both the *D. spathaceum* and *G. gasterostei* experiments, the population not naturally exposed to the parasite was substantially more susceptible to infection, which may have resulted from reduced selection to maintain or develop resistance. There was also some variation in resistance to *D. spathaceum* and *G. gasterostei* within-populations. The common garden design of the infection experiments implies that

this variation is partly under genetic control, thereby providing evidence for the potential of parasite-mediated selection.

Correlations between natural parasite abundance and experimental resistance were generally noisy, but there was a weak but positive correlation between resistance to *G. gasterostei* and natural abundance of *G. arcuatus*, indicating that variation in resistance to this parasite may be the result of divergent *Gyrodactylus*-mediated selection. In Chapter 5 and 6 I also documented considerable spatial variation in a component of the innate immune system, the respiratory burst response. The respiratory burst tended to increase with natural abundance of *D. spathaceum*, suggesting that divergent *Diplostomum*-mediated selection may drive investment in this measure of innate immune response. However, I found no correlation across populations between respiratory burst and resistance to *D. spathaceum*. Therefore, the respiratory burst response appears to be a poor indicator of resistance to this parasite. Lastly, in Chapter 7 I carried out a growth experiment and recorded significant spatial variation in juvenile growth rate, an important component of fish fitness. Integrating data from the infection experiments and the growth experiment, I found no evidence for a genetic trade-offs at the level of full-sib families across populations between juvenile growth rate and *G. gasterostei* resistance, *D. spathaceum* resistance or the respiratory burst response, in spite of ample variation among populations in all four traits. This suggests that variation in these traits is probably maintained via mechanisms other than trade-offs.

The patterns of spatial variation in resistance differed among the three macroparasite species, as did their relationship with natural infection levels. This may stem from differences between the three parasites in their virulence (the harm inflicted on their hosts). Presumably, the more virulent the parasite, the stronger the selection to evolve resistance. Therefore, certain parasite species may be more likely to generate population divergence in host traits than others. Parasite life history certainly influences the potential of host-parasite coevolution (Barrett *et al.*, 2008). Although the effects of *S. solidus* and *D. spathaceum* infection on host fitness are well-established, further infection experiments should determine precisely how *Gyrodactylus* infection impacts host fitness.

It has been demonstrated theoretically and empirically that parasites can drive the diversification of single host populations (Summers *et al.*, 2003; Duffy & Forde, 2009), but less is known about how parasites facilitate divergence among host populations (Buckling & Rainey, 2002b). Here, I have shown that divergence in macroparasite resistance and the innate immune response is a common feature of stickleback populations on North Uist, and that this divergence may be the result of adaptation to the local rate of parasite exposure (measured as parasite abundance). This thesis adds to a small but growing body of work that highlights how adaptation to local parasite communities can shape investment in the immune response and parasite resistance (Lindström *et al.*, 2004; Kalbe & Kurtz, 2006; Bryan-Walker *et al.*, 2007; Scharsack *et al.*, 2007a; Corby-Harris & Promislow, 2008; Hasu *et al.*, 2009). More broadly speaking, it demonstrates how an understanding of geographic variation in host ecology can help interpret variation in traits underlying host-parasite interactions (Sadd & Schmid-Hempel, 2009). Because such information is scarce for vertebrate-macroparasite interactions, the data presented here provide a valuable addition to our knowledge of spatial variation in host-parasite interactions.

To obtain a more complete understanding of the impact of space on stickleback-macroparasite coevolution in the North Uist system, a number of other features of host-parasite interactions must be considered, four of which are described in detail below. First, common garden experiments can reveal genetic variation in parasite resistance and immune response within and among host populations, but they cannot determine the evolutionary significance of this variation in the wild. Since natural infection is shaped by variation in parasite exposure and host condition in addition to genetic variation in parasite resistance, it is important to distinguish the relative importance of all three factors. This has important consequences for the interpretation of relationships between natural infection and experimental resistance across populations. Second, environmental factors such as temperature and resource availability may influence the expression of parasite resistance. If genotypes from different host populations respond differently to parasite infection with changes in the environment and/or populations vary in abiotic environment, then the strength and direction of parasite-mediated selection may also differ among populations, which in turn can mediate population divergence. Third, experiments should incorporate spatial heterogeneity of parasite populations. Particular combinations of

host and parasite genotype can influence the outcome of host-parasite interactions. At the population level, host and parasite genotypes can interact via local (mal)adaptation, and these spatial patterns can impact the evolution and divergence of parasite resistance and the immune response. Lastly, we must seek to understand how host divergence resulting from parasite-mediated selection could eventually lead to host reproductive isolation and speciation.

8.1 What is the relative contribution of parasite resistance, parasite exposure and host condition to natural infection?

All three infection experiments were carried out under common garden conditions. Fish from different populations were maintained at identical temperatures and were exposed to a standardised dose of parasite from the same parasite population. The advantage of this method is that it allows me to ascribe, at least partially, any variation in resistance to genetic differences among populations, even when the precise mechanisms conferring resistance are not known. However, it remains to be seen whether the variation in resistance to the three macroparasite species that I observed among the five stickleback populations is repeatable under more natural infection scenarios, for example in transplant experiments. This relates to a general issue in the evolutionary ecology of host-parasite interactions, one that has received surprisingly little attention: what is the relative role of host genetics and exposure in determining natural infection status (Grosholz, 1994; Little & Ebert, 2000; Poulin, 2007b)? If populations that differed significantly in infection levels in a common garden exposure fail to show such differences in a natural exposure, it argues against the evolutionary (adaptive) significance of the variation in parasite resistance. Instead, it suggests that the rate of parasite exposure is more important in determining infection than host genetics (Scott, 1991; Karvonen *et al.*, 2004a; Jansen *et al.*, 2010). However, even in artificial infection experiments the infection dose, a proxy for exposure rate, can alter the relative resistance of different host genotypes (Ben-Ami *et al.*, 2008; but see Osnas & Lively, 2004).

This issue also reflects a limitation of the method I have used throughout this thesis of correlating natural infection with experimental resistance across populations. As I

emphasised in the discussion of Chapter 4, the sign of this relationship leads to opposite conclusions about the importance of host genetics in shaping infection levels in the wild. If the correlation is positive, it suggests that environment (i.e. parasite exposure) contributes more to infection, whereas a negative correlation suggests that host genetics (i.e. resistance) contributes more infection. In reality, the situation is likely to be considerably more complex, with both host resistance and parasite exposure shaping parasite distribution (Little, 2002; Wilson *et al.*, 2002). An interesting possibility is if the sign of the relationship between experimental resistance and natural infection differs within and across populations. For instance, if experimental resistance is positively correlated with natural infection across populations, it indicates that the local rate of parasite exposure (assumed to be a proxy for the strength of parasite-mediated selection) has shaped investment in parasite resistance. However, within a population, more resistant genotypes may still have lower infection levels than more susceptible genotypes. Then it is simply the case that populations exposed to a higher abundance or prevalence of a parasite species *on average* are more resistant than those populations with lower levels of infection.

The discrepancy between exposure and resistance highlights why natural infection status cannot be used to infer differential host resistance to parasites (Goater & Holmes, 1997). Another reason is that resistance to parasites is likely to be influenced by host condition. Hosts in worse condition may be more likely to become infected, which in turn may reduce host condition, creating a vicious cycle (Beldomenico & Begon, 2010). In fact, this effect may even override the contribution of genetic variation in host resistance (Krist *et al.*, 2004). Therefore, interpreting the relationship between natural infection levels and experimental levels of resistance across populations becomes very difficult indeed. Disentangling the relative role of resistance, parasite exposure and host condition in determining infection status of individual hosts, and how this relationship may vary across different host populations, strikes me as a particularly important and potentially fruitful area of further work because it integrates concepts from disease ecology, ecological immunology and host-parasite coevolution.

8.2 How does variation in the abiotic environment influence host-parasite interactions?

Parasite exposure forms just one component of the environment. It is becoming increasingly clear that small changes in abiotic environmental factors such as temperature and resource availability can dramatically alter the expression of parasite resistance and the costs of infection (Lazzaro & Little, 2009). In other words, the outcome of a host-parasite interaction may be context-dependent. The environment can have an overall effect on parasite resistance, independent of host genotype. For example, Lambrechts et al. (2006a) have shown that food quality (concentration of glucose) has a significant effect on the resistance of the mosquito *Anopheles stephensi* to the malaria parasite *Plasmodium yoelii yoelii*. However, host genotypes may also respond differently to infection in different environment, such that the relative fitness of host genotypes varies across environments. For instance, Mitchell et al. (2005) exposed clones of *Daphnia magna* to a bacterial parasite, *Pasteuria ramosa*, at 4 different temperatures and found that the rank order of resistance of clones changed with increasing temperature. Such host genotype-by-environment (GxE) interactions are commonly invoked as a mechanism maintaining genetic variation in traits within a population because, provided that the environment is variable, no genotype will be universally fit (Schlichting & Pigliucci, 1998). Nevertheless, the extent to which GxE maintains genetic variation in parasite resistance and immune response in animal-parasite interactions is only just beginning to be appreciated (Lazzaro & Little, 2009). GxE interactions are typically studied within populations, but they may be just as important in maintaining variation in parasite resistance *among* populations. If the abiotic environment differs among populations and the relative resistance of host genotypes from different populations changes with environment, then the strength and direction of parasite-mediated selection may vary in different environments (populations). Importantly, spatially heterogeneous selection associated with the environment could contribute to the genetic divergence of parasite resistance and immune response (Thompson, 2005; Laine & Tellier, 2008).

There are few studies that have examined the effects of the environment on parasite resistance in vertebrate-macroparasite interactions, and even fewer that test for the

presence GxE interactions. There is one exception that is relevant to this thesis. Kolluru et al. (2006) found that carotenoid availability (a measure of diet quality) had a significant effect on the resistance of male guppies, *Poecilia reticulata*, to *Gyrodactylus turnbulli*. Stickleback resistance to *Gyrodactylus gasterostei* may similarly depend on carotenoid availability. If stickleback populations on North Uist differ in resource availability generally, and carotenoid availability specifically, then variation in natural infection with *Gyrodactylus* in the wild may be governed partially by the environment. As I mentioned in Chapter 7, there is some anecdotal evidence that resource availability varies substantially among lochs on North Uist. The availability of carotenoids in phytoplankton certainly varies (A. MacColl, unpublished data). Therefore, this environmental factor may be important in modifying the outcome of stickleback-*Gyrodactylus* interactions in the North Uist system. This idea could be tested experimentally by conducting infection experiments at different but realistic levels of carotenoid/resource availability across a number of stickleback populations. Importantly, if GxE interactions exist, then variation in carotenoid availability may alter the direction and strength of *Gyrodactylus*-mediated selection, and potentially lead to the evolution of quantitatively different patterns of resistance among host populations. Similar environmental factors should be tested for an interaction with resistance to *Diplostomum* and *Schistocephalus*. Ultimately, reciprocal transplant experiments are necessary to partition the environmental and genetic components of variation in parasite resistance (Nuismer & Gandon, 2008).

8.3 How does genetic variation within and among parasite populations affect host-parasite interactions?

In this thesis I have focused exclusively on the evolution and divergence of host populations, neglecting the potential impact of variation in the parasite population on the expression of host resistance and the immune response: a standard parasite population (genotype) was used in all three infection experiments. However, variation in host infectivity and virulence among parasite genotypes may be equally as extensive as variation in parasite resistance among host genotypes. In some animal host-parasite systems specific combinations of host and parasite genotypes ($G_h \times G_p$) are known to strongly affect the outcome of host-parasite interactions (Carius *et al.*, 2001;

Mackinnon *et al.*, 2002; Lambrechts *et al.*, 2005). In fact, this specificity is a prerequisite for negative frequency dependent models of host-parasite coevolution (Agrawal & Lively, 2002). Interestingly, there is evidence for $G_h \times G_p$ effects in the stickleback-*Diplostomum pseudospathaceum* interaction. Rauch *et al.* (2006a) carried out a cross-infection experiment with five F2 full-sib stickleback families and five clonal lines of *D. pseudospathaceum* and found that the number of parasites established was dependent on the combination of host and parasite genotypes. This study provides a rare example of the *potential* for coevolution in a vertebrate-macroparasite interaction. It would be worthwhile conducting similar experiments within the five stickleback populations studied here to assess the existence and generality of host-parasite specificity. This may be particularly feasible for *Gyrodactylus*, since clonal lines of this parasite can be established by infecting individual fish with a single parasite from a donor fish.

Host-parasite specificity is usually dealt with at the within-population level. At the between-population level, $G_h \times G_p$ interactions are manifested as local adaptation of the host or parasite population (Kaltz & Shykoff, 1998; Greischar & Koskella, 2007). If a host population is locally adapted to its parasite population, it is unlikely to show universal resistance to allopatric parasite populations and *vice versa*. However, it is important to distinguish between host local adaptation to a parasite population and host adaptation to the local parasite community. Local adaptation is a genetic interaction between host and parasite populations, whereas host adaptation to the local parasite community constitutes a more ecological interaction, as it links the differences in the abundance and/or prevalence of a particular, or multiple, parasite species to (genetic) variation in parasite resistance and immune response across host populations. Both types of host adaptation can maintain geographic variation in host traits. In this thesis, I was interested in the latter type of interaction. As I have explained in Chapter 4-6, the approach of correlating natural infection levels and experimental resistance assumes that differences in parasite abundance and prevalence reflect differences in parasite-mediated selection.

It also assumes that all host populations are exposed to parasite populations that are genetically identical. This second assumption may be unrealistic, considering that spatial variation in host-parasite coevolution is likely to lead to the divergence of both

host and parasite populations. Nevertheless, for some parasite species this assumption seems justified. For instance, a recent study by Louhi *et al.* (2010) compared the genetic structure of *Diplostomum pseudospathaceum* cercariae collected from four different snail populations covering a geographical range of 300km. In spite of the large distance separating parasite populations, no genetic structure was detected (Louhi *et al.*, 2010). Therefore, parasite species with complex life cycles, whose dispersal is mediated by a highly motile definitive host (such as a bird), may be more genetically homogeneous than directly transmitted parasite species over the same geographical distance (Criscione & Blouin, 2004; Barrett *et al.*, 2008). Since North Uist is a small island (~300km²), this raises the possibility that North Uist stickleback populations encounter genetically similar parasite populations, at least for those parasite species with a bird as a definitive host (e.g. *Diplostomum gasterostei* and *Schistocephalus solidus*). Weak genetic differentiation among parasite populations may in turn facilitate host local adaptation rather than parasite local adaptation (Gandon *et al.*, 1996; Kaltz & Shykoff, 1998).

Local adaptation studies should therefore be carried out to gain a better understanding of how coevolutionary dynamics may vary in space. Reciprocal cross-infection experiments involving a number of stickleback populations and parasite populations can reveal spatial heterogeneity among parasite populations as well the circumstances in which the variation in resistance to *Gyrodactylus*, *Diplostomum* and *Schistocephalus* among host populations observed here is repeatable. However, local adaptation can only be detected in naturally coevolving host-parasite interactions, since host populations have to be exposed to both sympatric and allopatric parasite populations. In all three infection experiments I intentionally *removed* the possibility of close coevolution between host and parasite populations by using either a non-native parasite species (*Gyrodactylus gasterostei* instead of *Gyrodactylus arcuatus*, and *Diplostomum spathaceum* instead of *Diplostomum gasterostei*) or a non-native parasite population (*Schistocephalus solidus* from Nottingham as opposed to from North Uist), to obtain a general overview of spatial variation in parasite resistance and the immune response. Local adaptation studies can complement this approach and offer an insight into the evolutionary significance of any observed variation among populations in host traits.

8.4 Can parasites drive reproductive isolation of host populations?

Ultimately, divergent selection exerted by parasites may lead to reproductive isolation and host speciation. This idea was first formulated by Haldane over 60 years ago (Haldane, 1949), but the link between parasites and host speciation is still poorly understood (Summers *et al.*, 2003). In recent years, it has been well-established that ecologically-based divergent selection can drive speciation (Schluter, 2000; Rundle & Nosil, 2005). The majority of work on this topic has focused on the role of competition (Pfennig *et al.*, 2007) and predation (Rundle *et al.*, 2003; Nosil & Crespi, 2006). However, given their ubiquity and strong effects on host fitness, parasites constitute an equally important agent of selection. The results in this thesis illustrate why the three-spined stickleback forms a good model for testing hypotheses of parasite-driven reproductive isolation: there are numerous replicate populations that harbour different parasite communities, there is pronounced population divergence in host resistance and immune response, and life histories are extremely variable. A few studies have attempted to identify traits mediating parasite-driven reproductive isolation in sticklebacks (Eizaguirre *et al.*, 2009a; MacColl, 2009b; Matthews *et al.*, 2010a). Functional traits that are under both divergent natural selection and divergent sexual selection ('magic traits') form particularly strong candidates because they can, in theory, accelerate speciation (Gavrilets, 2004).

Two putative 'magic traits' in sticklebacks are body size (MacColl, 2009b) and MHC genotype (Eizaguirre *et al.*, 2009a; Matthews *et al.*, 2010a). Body size is known to be under strong divergent natural selection (Nagel & Schluter, 1998; Bolnick & Lau, 2008) and appears to form the basis for assortative mating in several stickleback systems (McKinnon *et al.*, 2004). Similarly, MHC diversity may be the product of divergent natural selection (Wegner *et al.*, 2003) and has been shown to play a role in mate choice (Reusch *et al.*, 2001). Provided that parasites affect the evolution of either body size, MHC diversity, or both, then divergent parasite-mediated selection may eventually lead to reproductive isolation. This is another one of the reasons why I was motivated to examine the correlation between juvenile growth rate and parasite resistance: if increased investment in resistance is associated with slower growth, then parasites have the potential to drive reproductive isolation in the North Uist system,

so long as assortative mating is based on body size. Unfortunately, no such pattern was found. It would be interesting to follow up the work in Chapter 7 by investigating the relationship between growth rate and parasite resistance across more stickleback populations. Furthermore, reciprocal mate choice experiments should be carried out to determine which traits mediate assortative mating in the North Uist system. Given the dramatic differences between populations in adult size of wild-caught sticklebacks (A. MacColl, unpublished data), body size forms a strong candidate. Spatial variation in MHC diversity should also be characterised (Wegner *et al.*, 2003; Matthews *et al.*, 2010a).

Assortative mating based on body size or MHC is a form of pre-zygotic reproductive isolation (Coyne & Orr, 2004). Parasites could also drive post-zygotic reproductive isolation. For example, if hybrid genotypes are less resistant to parasites (have lower fitness) than either locally adapted parental genotype (Mouliat, 1999; Summers *et al.*, 2003), selection against hybrids will reinforce the genetic differences between parental populations (Schluter, 2000). Artificial infection experiments incorporating parental and hybrid crosses may help understand the quantitative genetic basis of parasite resistance and reveal whether hybrid genotypes suffer from lower resistance. Since divergent sympatric and allopatric stickleback populations can be readily hybridised, this is a feasible approach to studying the role of parasites in post-zygotic reproductive isolation. However, hybrid susceptibility is by no means the dominant pattern in studies that have compared parasite resistance of parental and hybrid genotypes from: hybrid resistance (heterosis) and hybrid dominance are also common (Mouliat, 1999; Wolinska *et al.*, 2008). The latter two scenarios may be less likely to facilitate speciation in rapidly diverging populations. A final mechanism through which parasites can contribute to reproductive isolation is selection against migrants, and this has been demonstrated experimentally in one saltwater stickleback population on North Uist (MacColl & Chapman, 2010). However, in general, empirical tests of the link between parasite-mediated selection and host reproductive isolation are lacking. This is an underexplored area of research that offers exciting opportunities to study the role of parasites as agents of diversification.

8.5 Concluding remarks

The comparative approach I have adopted in this thesis has shed some light onto the ecology and evolution of stickleback-macroparasite interactions specifically, and vertebrate-macroparasite interactions generally. Above all, it has highlighted the importance of considering spatial variation in host traits when studying parasite-mediated selection. However, as emphasised above, there are many important issues that remain to be addressed. Perhaps the most pressing of these are how variation in host traits, such as parasite resistance and the immune response, observed in the lab translates into fitness differences among hosts in the wild, and how variation in the abiotic environment influences the expression of parasite resistance among populations. Incorporating multiple, geographically isolated host populations and their parasite populations into future studies will undoubtedly help with this task.

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